UNIVERSITY OF SOUTHAMPTON

An Investigation of Potential Immunological and Metabolic Indices of Environmental Water Quality in the Shore Crab, *Carcinus maenas*.

by

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ABSTRACT

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AN INVESTIGATION OF POTENTIAL IMMUNOLOGICAL AND METABOLIC INDICES OF ENVIRONMENTAL WATER QUALITY IN THE SHORE CRAB, Carcinus maenas.

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The aim of this study was to investigate the potential of the shore crab, Carcinus maenas, to act as a biomarker of water quality using a range of metabolic and immunological indices of organism condition. Haemolymph total sugar, glucose, trehalose and hydrogen peroxide concentrations were measured to indicate the metabolic status of C. maenas whilst immunocompetence was inferred from haemocyte counts and phenoloxidase enzyme activities. Before any of these indices could be used to monitor changes in organism condition following exposure to artificial stressors the responses of these indices to natural environmental perturbation were recorded.

Baseline variability associated with tidal cycles was assessed in a computer controlled tidal tank with an effective excursion of 0.9m. Entrainable circatidal rhythmicity was demonstrated in the indices of total haemolymph sugar, hydrogen peroxide, haemocyte population and for the first time rhythms in the activity of the phenoloxidase enzyme were recorded. Furthermore the trends associated with tidal height were completely reversed when crabs were subjected to aerial exposure during low water.

No predictable cycles were recorded for any of the indices associated with the seasonal cycle, probably because of the number of environmental variables operating simultaneously. However, a significant positive correlation was seen between salinity and total haemocyte count possibly as a function of osmotic requirements and a positive correlation between bacterial load and phenoloxidase enzyme activity was recorded for the first time.

Having defined baseline variability, the responses of C. maenas to a pathogenic infection with Listonella anguillarum and a 50% dilution of the water soluble fraction of Wyche Farm crude oil were investigated. Significant and predictable decreases in the circulating haemocyte population were recorded following exposure to both stresses. Very little change in the metabolic status was seen following exposure to bacterial infection or organic pollution. Predictable decreases in the activity of the phenoloxidase enzyme were seen following bacterial infection which were followed by a recovery suggesting the possibility of immunostimulation. Hydrocarbon exposure appeared to have no affect on the phenoloxidase enzyme system in isolation. Synergistic exposure to hydrocarbon pollution and bacterial infection also produced very little change in the metabolic status of C. maenas but again produced predictable decreases in the circulating haemocyte population. Combined exposure to the two stressors seemed to prevent the phenoloxidase enzyme system responding to the presence of a bacterial infection and the idea of immunosuppression of the phenoloxidase system was suggested for the first time in marine crustaceans.

The potential use of C. maenas as a biomarker of water quality and the value of the majority of the indices tested was questioned because of their inherent resilience to the stresses imposed.

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CHAPTER ONE

GENERAL INTRODUCTION

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1.1 WATER QUALITY.

The term water quality is a widely used expression which has a broad spectrum of meanings dependent on the scientific viewpoint adopted (see reviews by Gower, 1980; Maxwell, 1983; and Tebbutt, 1992;). What is deemed as a "good water quality" by marine ecologists may be seen as insufficient by human health experts. Tebbutt (1992) has put forward the view that the quality of a body of water depends upon a range of biological, physical and chemical factors including: odour, colour, taste, temperature, turbidity, particulate load, dissolved gases, organic and heavy metal pollutant loads and bacterial populations. As suggested above, not all of these parameters are of interest depending on the scientific standpoint; for example the colour and taste of a body of water makes little difference to the organisms found therein but is of aesthetic interest to man.

According to Phillips (1980) the necessary monitoring of water quality can be achieved using two approaches - firstly by analysing the water itself and secondly by examining the condition of the organisms present. Water analysis tends to be costly, involving sophisticated sampling and preconcentration of samples prior to analysis; further to this the low level of many pollutants in water bodies also gives rise to problems of analytical sensitivity as well as increasing the possibility of sample contamination. Even if these problems are dealt with a remaining problem lies in the fact that the presence of a prospective pollutant in a water body does not necessarily indicate that it is bioavailable and hence it may have a very limited influence on the ecosystem as a whole.

1.2 CONCEPT AND USE OF BIOMARKERS FOR WATER QUALITY MONITORING.

Phillips (1980) has suggested that the use of organisms to monitor changes in water quality does have its merits. They tend to be cheaper to sample and they have the ability to bioaccumulate toxins, for example: mercury, cadmium, copper, lead, radionuclides, DDT and other halogenated hydrocarbons (see review by Clark, 1989) facilitating their detection. One of the key attractions of biomarkers is however that they reflect the presence of only those toxins which are bioavailable and as a result they reflect an ecological consequence of a pollutant rather than just its presence or absence. In the first instance the deterioration in condition of a biomarker, assessed using a variety of indices, can be used as cheap and rapid indicator of falling water quality from which more specific determinations can be made.

The choice of biomarker is an important consideration in any study, Phillips (1977) has reviewed the selection of biomarkers for monitoring heavy metal pollution in coastal waters. From his work eight basic prerequisites can be drawn up relating to biomarkers in general as summarised below:

1) The organism should accumulate the pollutant without being killed by the levels encountered in the environment.

2) The organism should be sedentary in order to be representative of the study area.

3) The organism should be abundant throughout the study area.

4) The organism should be sufficiently long lived to allow the sampling of more than one year class, if desired.

5) The organism should be of reasonable size, giving adequate tissue for analysis.

6) The organism should be easy to sample and hardy enough to survive in the laboratory, allowing defecation before analysis (if required) and laboratory studies of pollutant uptake.

7) The organism should tolerate brackish water.

8) All organisms of a given species used in a survey should exhibit the same

correlation between their pollutant content and the average pollution concentration in the surrounding water, at all locations studied, under all conditions.

Bayne *et al.* (1985) have since also suggested that estuarine organisms are of more value. Estuaries are naturally testing environments and their inhabitants often possess a wide physiological tolerance to perturbation, using the terminology of Fry (1947) "their zone of resistance is great". Bayne *et al.* (1985) argue that such eurytopic individuals allow for easier recognition of deleterious environments, through the measurement of altered physiological or biochemical steady states, than stenotopic organisms that have a limited tolerance. It is conceivable however, that this theory may also be equally valid in reverse. If eurytopic organisms are able to accommodate changes in water quality without any significant cost they may prove to be too insensitive for monitoring purposes - the theory remains inconclusive.

The above discussion has focused on the use of organisms to monitor changes in water quality due to both natural perturbation as well as the influences of mankind (i.e. pollution). What is often measured when using biomarkers is an index of organism condition or stress index and it is impossible to proceed further without first defining the term stress and considering its implications. Despite many definitions the use of the term stress remains vague; indeed even Seyle (1950) who was perhaps the first to categorise mammalian responses to environmental change in terms of his: "General Adaptation Syndrome," failed to completely define his widely used term "stress." Since that time, in an effort to ensure conformity amongst workers, several authors have offered definitions for the term stress as quoted below:-

a state produced by an environmental stimulus or other factor which extends the adaptive responses of an animal beyond the normal range, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced. (Brett, 1958).

It is important to note that this definition implies that an animal's evolved

adaptive ability is being stretched to a point where damaging effects on organism fitness result. This definition has since been superseded by Bayne (1984) with:-

the environmental stimulus which, by exceeding a threshold value, disturbs normal animal function.

- which emphasises the causative agent of an organism's change in condition rather than the response itself and which was offered by Bayne (1984) as an alternative to provide equivalence with the definition of ecosystem stress as defined by Odum (1967). A final definition offered by Barton (1992, pers. comm.) returns the emphasis back to the animal's response to perturbation:-

a non specific response of a body to any demand placed upon it such that it causes an extension of a physiological state beyond a normal resting state.

It must be pointed out that these last two definitions offered for the term stress do not necessarily imply detriment to the organism or organisms concerned (suggesting that some forms of stress may have a positive influence on organism fitness) and that all three definitions do contain vague terms in themselves. For example the definition of a "normal state" is problematic - should the physiological and biochemical responses to the extreme environmental variation associated with the seasons be regarded as stress responses or merely within the "normal repertoire" of an organism? Theoretical arguments such as these are beyond the scope of this introductory chapter and are of little consequence in monitoring programmes as long as they are accounted for when conclusions are being made.

In accordance with the guidelines suggested by Phillips (1977) studies of biological response to environmental variability have, in the past, tended to use ubiquitous, hardy and sessile bivalve species (for example *Mytilus edulis*: Mullin & Riley, 1956; Segar *et al.*, 1971; *Pecten maximus*: Segar *et al.*, 1971; *Mercenaria mercenaria:* Raymont (1972); and *Crassostrea gigas*, Boyden &

Romeril, 1974) or macroalgae (for example: Rhodophyceae: Leatherland & Burton, 1974; and Chlorophyceae and Phaeophyceae: Mullin & Riley, 1956). With this present study it is aimed to expand on previous efforts to include the crustaceans, using the common shore crab, *Carcinus maenas* (L.), as a model organism. The choice of *C. maenas* meets all the requirements as laid out by Phillips (1977) except the second; crustaceans are, by nature, mobile organisms and so flout the second prerequisite. Their consideration is however, justified as they are commercially viable and represent a different set of biochemical and immunological pathways which will respond differently to any environmental disturbance such as pollution; they are also affected by a different suite of diseases (Chisholm, 1993). Further to this due to the over exploitation of natural stocks commercially valuable crustacean shellfish are increasingly being reared in enclosed aquaculture systems where their ability to move away from stressful situations is impeded. For example: prawns and shrimps for human consumption in Ecuador (Rosenberry, 1983) as well as Japan (Bardach *et al.*, 1972). The blue crab *Callinectes sapidus* is also widely raised as a food source (Costlow & Bookhout, 1959; see review by Provenzano, 1985). Similarly Kittaka & Booth (1994) have discussed the requirements and prospects for the commercial aquaculture of spiny lobsters (Crustacea: Decapoda: Palinuridae).

1.3 INDICES OF ORGANISM CONDITION (*STRESS INDICES*).

The choice of biomarker must however, be seen as only part of the problem. An organism occurs at the "interface" between the environment and the cell, suffering any stresses imposed by that environment and acting as a carrier or medium in which an index of physiological, biochemical or immunological stress can express itself (for example elevated respiration or excretion.) Considerable time and thought must be devoted to the choice and suitability of a particular index. Needless to say the number of suggested indices is great and have been extensively reviewed by: McIntyre & Pearce (1979), Bayne *et al.* (1985), Giam & Ray (1987), Abel & Axiak (1991), and Peakall (1992).

Bayne *et al.* (1985) have proposed that the different indices of organism

condition can be classified into two groups: general and specific. General indices are applicable, irrespective of the nature of the stress, and reflect the general condition of the biomarking organism whilst specific indices tend to be suitable to a single type of stress, for example bacterial infection or organic pollution. The variety of stress indices available can alternatively be subdivided according to the classes physiological, biochemical and cytological. The variety of different indices used in the past has been summarised in table 1.01 in which the original workers and review papers of each index have been indicated.

In this present study a novel suite of parameters are to be measured to give an indication of the metabolic status of *C. maenas* as well as the immunocompetence of the crab. Measures of haemolymph total sugar, glucose, and trehalose as well as haemolymph hydrogen peroxide are to be made to give and indication of the metabolic condition of the organism. At the same time data collected on haemocyte number and the activity of the immune defence enzyme phenoloxidase will provide information on the immune status of *C. maenas*. As with all research programmes the assays are developed and refined throughout the duration of the study and some of the methodologies are continually modified as recorded in this thesis. Further to this a measure of *in vitro* cell stability (neutral red retention assay) is investigated in relation to studies on the influences of petroleum hydrocarbon pollution. All of the biochemical and immunological parameters measured throughout this research are discussed more completely below.

1.3.1 HAEMOLYMPH SUGAR CONCENTRATIONS.

Elevations in the level of circulating haemolymph sugars (hyperglycaemic events) in response to stress in invertebrates have been reported for many years; initially by Morgulis (1922, 1923) who noted a decrease in the concentration of reducing metabolites in *Panulirus argus* with acclimation to the laboratory environment and Roche & Dumazert (1935) for the crab, *Cancer pagurus,* suffering emersion stress. Similarly Abramowitz *et al.* (1944) have reported

GENERAL	SPECIFIC
PHYSIOLOGICAL: *Scope for Growth*: Warren & Davis, 1967; Widdows & Bayne 1971; Bayne *et al.*, 1985 *Oxygen:Nitrogen Ratio*: Corner & Cowey, 1968; Mayzaud, 1973 *Growth Efficiency*: Ivlev, 1961; Widdows *et al.*, 1981 *Body Condition Index*: Baird, 1958; Walne, 1970; Bayne & Thompson, 1970	None
BIOCHEMICAL: *Adenylate Energy Charge*: Atkinson & Walton, 1967 *Taurine:Glycine Ratio*: Jefferies, 1972 *Haemolymph Proteins*: reviewed by Tripp & Fries, 1987 *Enzyme Activities*: reviewed by Tripp & Fries, 1987	*Mixed Function Oxidases*: Payne & Penrose, 1975; Pedersen *et al.*, 1976; reviewed by Bayne *et al.*, 1985 *Metallothioneins*: Winge *et al.*, 1975 *Steroid metabolism*: Freeman *et al.*, 1982
CYTOCHEMICAL/HISTOLOGICAL: *RNA/DNA Ratio:* Bulow, 1970; Keil *et al.*, 1971; McKee & Knowles, 1986 *Neutral Red Retention Assay*: Borenfreund & Puerner, 1985; Reader *et al.*, 1989	*Formation of DNA Adducts*: Shugart *et al.*, 1983 *Dimethylthiazoldiphenyltetrazoliumbromide (MTT) assay*: Mosmann, 1983

TABLE 1.01 Summarising adopted indices of organism condition. Cited authors represent original workers and reviewers of the use of each index.

hyperglycaemia following overcrowding in the blue crab, *Callinectes sapidus;* and Kleinholz & Little (1949) have reported haemolymph hyperglycaemia following experimentally induced asphyxia in specimens of the spider crab, *Libinia emarginata*. More recently Telford (1968) has demonstrated an increase in the concentration of reducing metabolites within the haemolymph of the lobster, *Homarus americanus*, following handling stress.

C. maenas is a shallow water species (occurring from the supralittoral down to depths of approximately sixty metres, Fish & Fish, 1989) that is subjected to the influence of tides and the hydrostatic pressure changes associated with them. As has been extensively reported by Naylor (1988); Reid & Naylor (1989); McGaw & Naylor (1992); and Warman *et al.* (1993); much of the behaviour (including foraging and tidal migration) of *C. maenas* demonstrates circatidal rhythmicity. As a consequence many metabolic parameters have also been shown to have circatidal rhythmicity. Rajan *et al.* (1979) using freshly caught specimens and Williams (1985) using laboratory maintained individuals have both reported fluctuations in haemolymph anthrone responsive sugar concentrations (total mono - and oligosaccharides) with respect to tidal elevation with high concentrations being recorded at low water.

From the above discussion it can be seen that changes in the level of sugar in the haemolymph of crustaceans can indicate the imposition of some form of stress but that any conclusion must be made in the light of other impinging variables such as tidal state or season.

1.3.2 HAEMOLYMPH GLUCOSE CONCENTRATIONS.

Characteristically glucose has been reported as the major reducing sugar which responds to stress (Telford, 1968; Ram & Young, 1992) and it has been concluded that the hyperglycaemic response to stress exhibited by crustaceans is due to the conversion of carbohydrate from a storage compound such as glycogen to a more metabolically available substrate. In terms of glucose alone Dean & Vernberg (1965) have reported an increase in specimens of *Uca pugilator* suffering thermal stress (increasing from values of approximately 6.5mg % at 2°C to

29.0mg % at 30°C) and Telford (1974b) has demonstrated elevated levels in the crayfish *Orconectes propinquus* and *Cambarus robustus* following exposure to the halogenated aromatic: dichlorophenol. More recently Hansen *et al.* (1992) have recorded a six fold increase in the haemolymph glucose of *C. maenas* following a week's exposure to copper (II) chloride at a concentration of 10ppm. As with any indicator fluctuations in the level of glucose in response to imposed stress are superimposed on any natural variability. Stott (1932, cited in Kleinholz & Little, 1949) was perhaps the first to demonstrate this - showing a high concentration of glucose in the haemolymph of newly moulted crustaceans. The later work of Telford (1974a) showed fluxes in the concentration of blood sugar with respect to moult cycle in the crayfish *O. propinquus* and *C. robustus* and demonstrated an elevation in blood glucose (4.2-4.5 $mg.100ml^{-1}$) of both males and females during premoult stage D, (as defined by Drach (1939)) followed by a rapid decline during ecdysis (to 1.3-1.6 $mg.100ml^{-1}$) before a recovery during intermoult.

Seasonal variations in blood composition have also been recorded. Telford (1974a) reported a rise in blood sugar during the summer, reaching a peak (5.7-5.9 $mg.100ml^{-1}$) in July and Kucharski & Da Silva (1991) have reported elevated glucose levels in the estuarine crab (*Chasmagnathus granulata*) during the summer and winter (approximately 11-12 mg. $100ml^{-1}$ for both seasons).

As with haemolymph total sugar it can be seen that elevations in the concentration of glucose may be indicative of stressful environments but, as before, caution must be exercised due to the natural variation in concentration associated with seasonal cycles and physiological processes such as moulting and reproduction.

1.3.3 HAEMOLYMPH TREHALOSE CONCENTRATIONS.

The role of the disaccharide sugar trehalose (an α (1-1) dimer of glucose) in invertebrate metabolism is somewhat enigmatic. Telford (1968) reported that following brief periods of handling lobsters, *H. americanus,* there was a removal of trehalose from the circulation. Whilst the evidence was not entirely conclusive it was suggested that the oligosaccharide was acting as an intermediate reserve for

glucose. More recently Clegg & Jackson (1992) have reported trehalose synthesis in *Artemia franciscana* embryos following heat shock which led them to suggest that trehalose may have a protective role at a cellular level possibly by being involved in the maintenace of membrane stability or by preventing the denaturation of proteins at high temperatures.

Research into the potential roles of trehalose has been much more widespread at a microbiological level (see Feofilova, 1992). Evidence has been published to suggest that it has a role either in the formation of heat shock proteins (HSPs) or working in conjunction with HSPs in fungi; as a carbon reserve and as a lipid protector in cell membranes. Finally, trehalose derivatives such as the reduced form of lysodectose have been shown to "trap" toxic oxygen radicals (O_2^-) and hydroxide radicals (OH•) in the fungi *Cunninghamella japonica* (Tereshina *et al.*, 1991; reviewed in Feofilova, 1992).

1.3.4 HAEMOLYMPH HYDROGEN PEROXIDE CONCENTRATIONS.

Hydrogen peroxide occurs in marine invertebrates as a metabolic end product of routine metabolism and following the production of superoxide ions.

The production of superoxide ions as a non specific immune response has been demonstrated in a variety of molluscs for example the scallop, *Patinopecten yessoensis,* (Nakamura *et al.*, 1985); the pond snail, *Lymnaea stagnalis*, (Dikkeboom *et al.*, 1987); and the American hard clam, *Mercenaria mercenaria*, (Hawkins *et al.*, 1993) for which it is considered to be a major humoral defensive mechanism. Oxygen free radicals are damaging to organic material and have the ability to disrupt cell membranes and organelles including those of invading pathogens.

In crustaceans the role of hydrogen peroxide and the determination of superoxide production has proved more elusive. Certainly the production of hydrogen peroxide via routine metabolism is accepted but disagreement has occurred over its role as a defence agent.

White *et al.* (1985) reported from their studies that there was little evidence

to support the view that superoxide production occurred to any extent within the Crustacea. However, the recent work of Bell & Smith (1993) has shown superoxide production in isolated preparations of hyaline cells incubated in the presence of a variety of elicitors including lipopolysaccharides, phytohaemagglutinin and concanavalin A.

With respect to haemolymph glucose any measurements of hydrogen peroxide concentration must be interpreted in the light of all impinging environmental variables. An organism's metabolism will vary throughout both the year and, for crustaceans, the moult cycle and whilst no measurements have been reported it is inevitable that hydrogen peroxide concentrations will fluctuate. Hawkins *et al.* (1993) have recorded tidally associated rhythms of hydrogen peroxide concentration within the haemolymph of the American hard clam, *Mercenaria mercenaria*, with elevated concentrations (6mg. per litre) being recorded just after low water and low concentrations (approximately 0.5mg. per litre) around high water. These workers concluded that this variation reflected a fluctuating level of immunocompetence throughout the tidal cycle. Recently Delaney (1991) has recorded elevations in hydrogen peroxide at low water for *Carcinus maenas* tentatively concluding that this also demonstrated a molluscan type variability in immunocompetence.

1.3.5 HAEMOCYTE POPULATION.

Fluctuations in the total number of haemocytes and in the proportions of different classes of haemocyte following stress have been recorded by a number of workers. Truscott & White (1990) have demonstrated an increase in the total haemocyte population (THC) in *C. maenas* following a gradual increase in temperature from 10°C to 20°C; Hawkins *et al.* (1993) have shown an elevation in the population of small amoebocytes in specimens of *M. mercenaria* suffering aerial exposure whilst Delaney (1991) has reported decreased haemocyte populations for shore crabs subjected to an identical stress.

The aggregation of haemocytes following infection with bacterial and viral pathogens has been reported on a number of occasions (Spindler Barth, 1976;

Smith & Ratcliffe, 1980a&b). Aggregation results in the removal of haemocytes from circulation and an apparent drop in the cell population as has variously been recorded by: Bang (1971) and White *et al.* (1985) for *C. maenas*; and Plytycz & Seljelid (1993) for the sea urchin, *Strongylocentrotus droebachensis*.

The classification of invertebrate haemocyte classes has in the past proved difficult. Cheng (1981) was forced to conclude for bivalves that a variety of interpretations as to how many types of haemocytes exist with little agreement as to the designation of these cell types. Such a picture is also true for crustaceans with a number of classification schemes being proposed including those of Williams & Lutz (1975); Smith & Ratcliffe (1978); and Hose *et al.* (1990). Despite the recommendation made by Hose *et al.* (1990) that any classification scheme should include not only aspects of morphology but also cytochemistry and function, three morphologically separate classes are generally accepted today: granular cells, semigranular cells (together termed granulocytes); and agranular cells or hyalinocytes.

1.3.6 PHENOLOXIDASE ENZYME ACTIVITY.

Phenoloxidase (EC.1.14.18.1) has been defined (Aspan & Söderhäll, 1991) as a "bifunctional copper containing enzyme, which catalyses both the *o* - hydroxylation of monophenols and the oxidation of diphenols to quinones".

The distribution of phenoloxidase throughout the arthropods and its role in cuticular tanning and melanisation has been extensively reported (see Krishnan, 1953; Decleir & Vercauteren, 1965; and Summers, 1968). However, following the discovery that invading pathogens are often melanised within the haemocoel of arthropods (Söderhäll & Ajaxon, 1982) and that the zymogen prophenoloxidase can be elicited into is active form by minute amounts of bacterial lipopolysaccharides (Söderhäll, 1982) workers have investigated the possibility that phenoloxidase may act as an immune defence agent being involved in the recognition of foreign particles.

Since these initial studies a large volume of data has been generated concerning the activation of the phenoloxidase enzyme and the mechanics of the

cascade and also the potential roles of phenoloxidase constituting an immune response. Söderhäll & Smith (1983) using density gradient centrifugation on Percoll demonstrated the presence of prophenoloxidase in the granulocytes of crustaceans concluding that prophenoloxidase is released into the plasma whereupon it is activated by glycans including laminarin and zymosan via a serine proteinase in the presence of calcium ions (see also the more recent work of Aspan & Söderhäll, 1991). Smith & Söderhäll, 1991 have also provided evidence to show the presence of phenoloxidase throughout the marine arthropods and other invertebrates including: molluscs, annelids, echinoderms and urochordates.

These and other observations have been summarised in figure 1.01 for clarity - reviews may be found in Söderhäll & Smith, 1986; and Söderhäll, 1992. From figure 1.01 it can be seen that endogenous inhibitors are present within invertebrates which under normal circumstances inhibit the activation of the phenoloxidase system (Tsukamoto *et al.*, 1992). Further to this it is evident that the active phenoloxidase enzyme has a number of roles constituting an immune defence. These defences are either brought about by the direct action of phenoloxidase, for example melanisation and encapsulation (Zahedi *et al.*, 1992), or are mediated through the organism's haemocyte population (as in the case of spreading and phagocytosis). Further to this it can be seen that the phenoloxidase enzyme can itself induce the release of more zymogen by the granulocytes giving rise to a positive feedback. Just recently (Söderhäll, 1994; pers. comm.) has discounted the theory of an enzyme cascade as being misleading. Clearly the mechanics of invertebrate immune defence still require further research.

The use of enzyme systems as indicators of stress in marine organisms has been suggested in the past (see table 1.01 and reviews by Bayne *et al.*, 1985; and Tripp & Fries, 1987). One enzyme which has been used to indicate immunological stress is lysozyme. This enzyme is involved in the lysis of cell walls including those of invading pathogens. Hawkins *et al.* (1993) have reported fluctuating levels of lysozyme in *M. mercenaria* over tidal cycles and have suggested (using other supporting evidence) a variation in the immunocompetence of this neritic mollusc. To date however, no one has investigated the possibility of using the phenoloxidase enzyme system to indicate the condition of marine invertebrates and

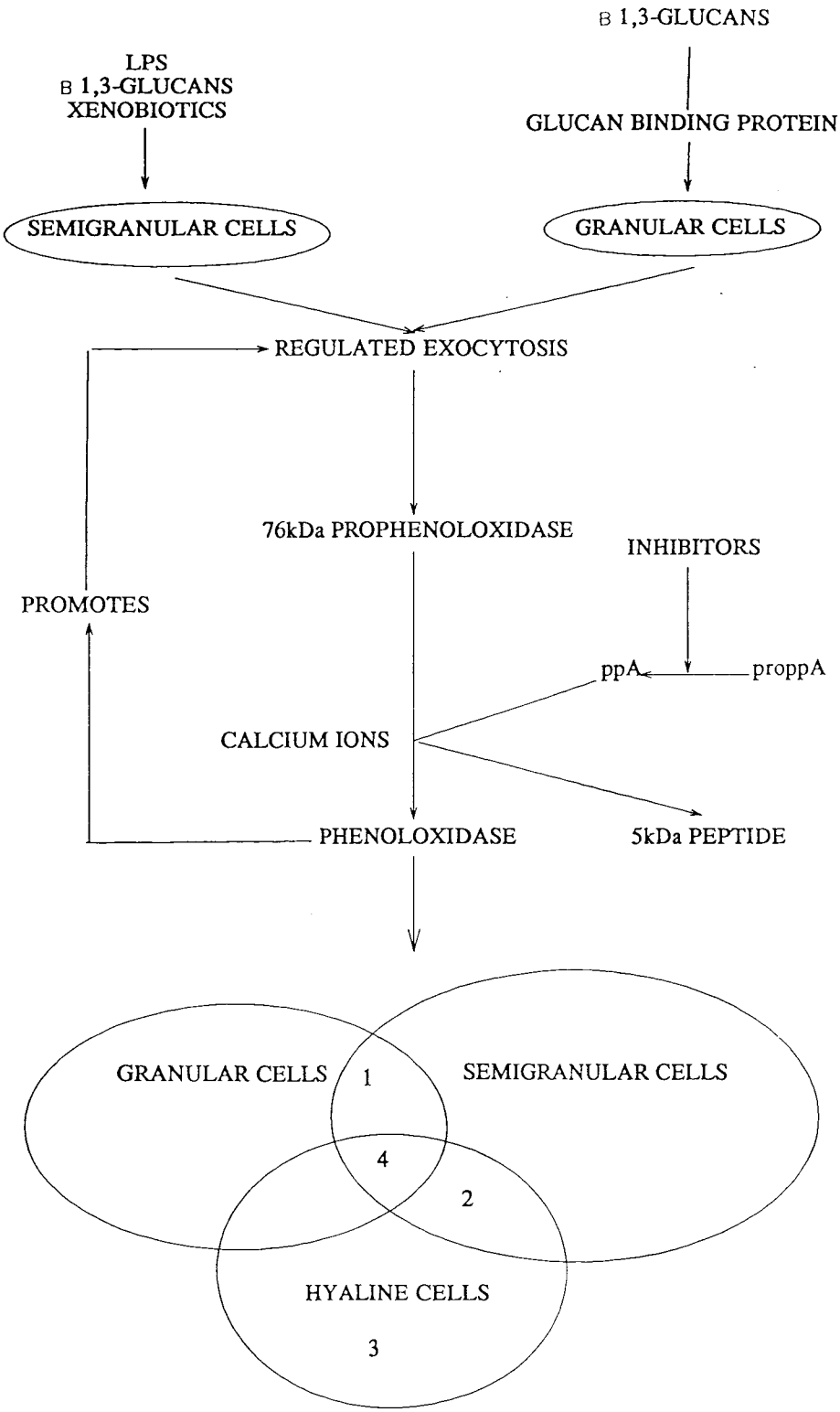

FIGURE 1.01 Summarising present theories relating to the phenoloxidase system as discussed in the text. LPS = Lipolysaccharide, proppA = inactive serine proteinase, ppA = activated proteinase. 1 = Lysis and release of cytotoxic products, 2 = Spreading and encapsulation, 3 = Phagocytosis, and 4 = Nodule formation. Demonstrated inhibitors of the activation of proppA include a 155kDa trypsin inhibitor and an α-2-macroglobulin like molecule. Note the requirement for calcium in the activation of the zymogen prophenoloxidase.

no work has been carried out to determine baseline variability in the level of this enzyme within organisms. One of the key aims of the present study was to investigate circatidal and seasonal variability in the activity of the immune enzyme phenoloxidase and to assess its value as an indicator of marine crustacean immunocompetence.

1.3.7 NEUTRAL RED RETENTION ASSAY.

In recent years there has been a conscious effort to move away from the use of animals in toxicity testing towards *in vitro* testing on cultured cellular material (Borenfreund & Puerner, 1985; Reader *et al.*, 1989; Dierickx & van de Vyver, 1991; Babich & Borenfreund, 1991; Martin *et al.*, 1994; and Korting *et al.*, 1994). To this end a number of workers have developed *in vitro* alternatives to the more usual whole organism bioassays of the past.

Borenfreund & Puerner (1985), who developed the neutral red uptake assay (NRU) noted that stressed cells did not take up as much of the supravital cationic dye neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) as did control cells concluding that this was due to a decrease in cellular membrane stability. Reader *et al.* (1989) modified the original assay into what has become known as the neutral red retention assay (NRR). After a defined period of dye loading they recorded the rate of loss of stain from the cells and noted that stressed cells lost their dye more rapidly than control cells..

Most recently Lowe *et al.* (1992) working with dab, *Limanda limanda*, hepatocytes have described a refinement of this assay which assesses the rate of dye release from loaded lysosomes within the cells.

The NRR and NRU assays have found wide application since development, being adopted by a number of workers using a variety of cell types and lines cultured *in vitro*. For example Babich & Borenfreund (1990) have comprehensively studied the affects of a range of toxins including organometals, cancer chemotherapeutics, surfactants, pharmaceuticals, pesticides and toluenes on a series of different cell cultures (rabbit corneal epithelial, mouse and hamster fibroblasts, mouse macrophages and human hepatomas); Babich *et al.* (1992) have

investigated the toxicity of methylated phenylenediamines using cultured mouse fibroblasts; Babich & Stern (1993) used hepatoma cell line HepG2 cultures and mice fibroblasts to determine the varying toxicity of different napthoquinone isomers; Valdivieso-Garcia (1993) has harvested vero cells from a variety of species to investigate the action of verotoxins produced by *Escherichia coli*; Korting *et al.* (1994) have used human skin cell cultures to determine the potential irritancy of surfactants; Kappus & Reinhold (1994) used human epidermal keratinocytes to assay the toxicity of selected heavy metals; and finally *Cyprinus carpo* brain cell cultures have been used to investigate the effects of chlorophenols (Saito & Shigeoka, 1994).

With this research it is hoped to modify the NRR assay to compare the release rates of haemocytes harvested from crabs subjected to pollutants *in situ* in a similar approach to that adopted by Lowe *et al.* (1992); Jacobson *et al.* (1993) who investigated toxicity in freshwater mussels (Bivalvia: Unionidae); and Schild *et al.* (1993) who investigated the *in situ* affects of alcohols on *Enteromorpha intestinalis* tissue.

Before any investigation of these indices' responses to a variety of imposed stresses their background variability arising as a result of natural environmental phenomena, such as tidal cycle and season, must be elucidated. As mentioned earlier for each of these parameters natural fluctuations have been reported and the magnitude and direction of the fluxes must first be assessed to ensure that they do not mask any changes due to artificially imposed stresses. The first two chapters of this thesis summarise investigations made concerning the degree of variability in the chosen indices with respect to tidal cycle and season. Once baseline fluctuations had been determined research focused on the affects of live bacterial infection on the shore crab (chapter 4) followed by investigations on the influence of oil pollution (chapter 5). Of course in a natural situation organisms face bacterial challenge and different forms of pollution in synergy and chapter 6 summarises research into the affects of pathogen challenge and organic pollution acting in concert.

1.4 STUDY AREA.

One of the attractions of *C. maenas* as a model crustacean is its availability. In spite of the reduction in numbers of crabs over the winter months as they move offshore to deeper water (Jensen, pers. comm.; a phenomenon also noted for the hermit crab, *Pagurus longicarpus*, by Rebach (1974)) experimental organisms were generally available throughout the year in the Solent region. Crabs were collected from two sites within the Solent (see figure 1.02); those collected for laboratory analyses of bacterial infection and organic pollution were collected from the littoral zone using baited pots (Delmar Engineering, Chichester) deployed over night on the mud flat system around Hayling Island whilst crabs used for baseline studies of seasonal and tidal associated variability were collected from the sublittoral using baited pots deployed in the River Itchen. One of the tenets of the present research was to investigate the *sublethal* effects of stress on organism condition. As a consequence very few individuals were destroyed and all live crabs were returned to the wild after use. All crabs, irrespective of collection site, were returned to the Hamble Estuary, a sufficient distance from both points of collection to avoid resampling individuals.

1.4.1 PHYSICAL AND INDUSTRIAL CHARACTERISTICS OF THE SOLENT AND SOUTHAMPTON WATER.

The Solent extends for approximately 20 miles along the south coast of England, and is protected to the south by the Isle of Wight. The depth tends to vary between 10 and 30m. Although the Solent regime is affected by the characteristics of the English Channel, the shallower water, bottom topography and land boundaries in the region also affect the tides. The Solent has a very complex tidal regime, the prominent feature of which is the double high water. The ebb tide is extremely short (3.75 hours) and tends to be vigorous with velocities which may be up to 100% greater than the flood velocities (ABP tide tables for Southampton).

Southampton Water itself was formed as a result of the joining of the Test and Itchen estuaries. This important U.K. port has a width of 2km at high water and is classed as a deep estuary being maintained, by dredging, at 10m.

Development of the estuary has continued for many years, reclamation of land first taking place in 1889 and continuing in the 1930's with the development of Princess Alexandra Dock and Dock Head. This development has inevitably led to the industrialisation of the entire estuary and two oil refineries are present today, one at Fawley dealing with imported crude oil and one on the River Hamble which exports refined oil piped as crude from the Wyche Farm oil field in Dorset (Parkhouse, pers. comm.). The western shore of Southampton Water is heavily industrialised and was the site of Monsanto Chemicals which, until its recent closure, was the sole manufacturer of polychlorinated biphenyls (PCBs) in the U.K.

From a hydrographic standpoint Southampton Water has a Hansen Rattray classification 2A (Robinson I., pers. comm.) indicating that it is well mixed with slight stratification. The double high water and short vigorous ebb discussed above results in the estuary remaining relatively "clean" despite the effluent input from local industry which amounts to 5.8×10^5 m^3 day^{-1} (Robinson, pers. comm.; 1993). The mean tidal amplitude of Southampton Water is 1.2m on the western shore and 3m on the eastern side.

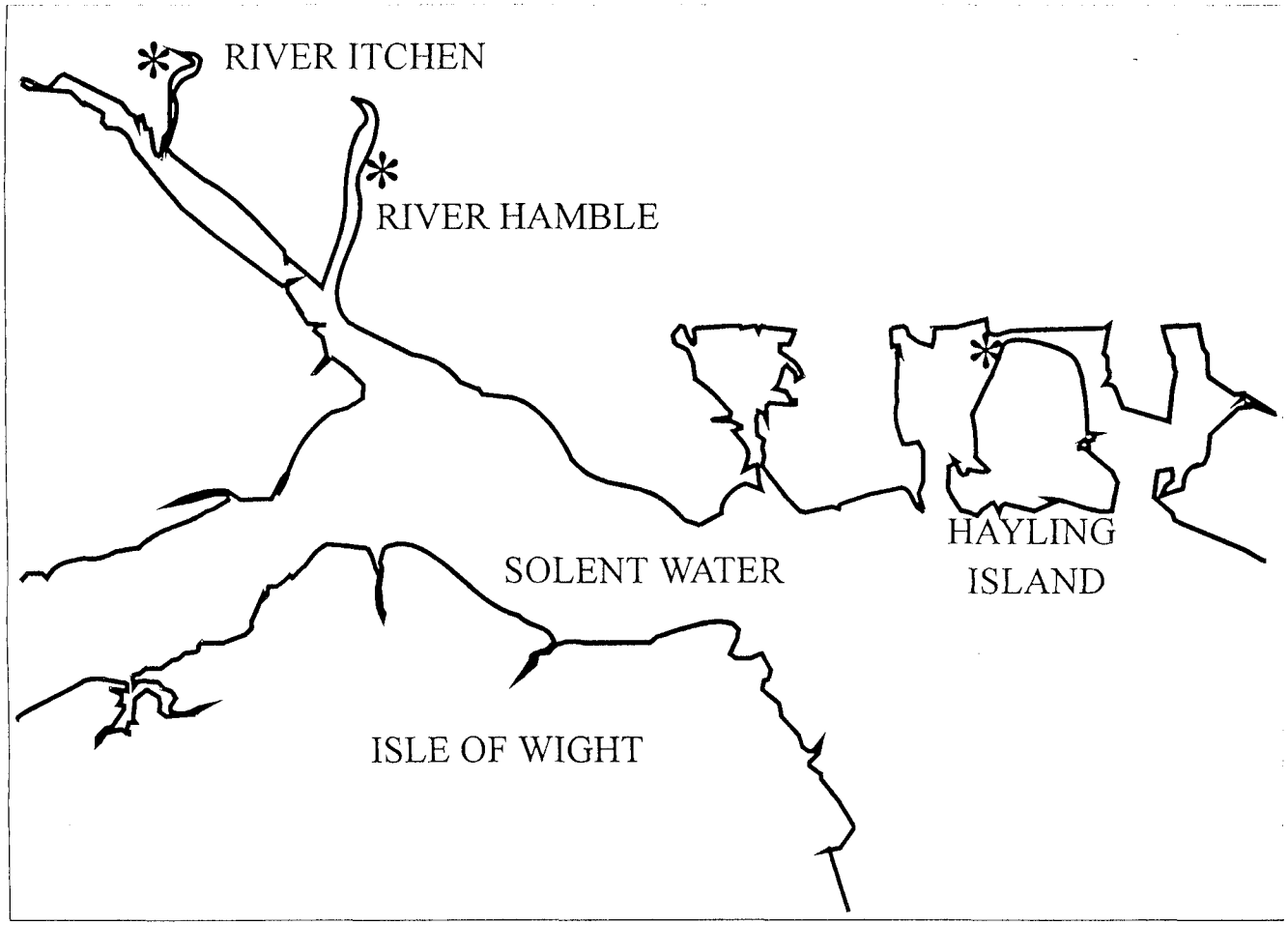

FIGURE 1.02 Figure showing the two collection sites (River Itchen and Hayling Island) and the release site (River Hamble) used throughout this study. The exact position of each site has been marked with an asterisk.

CHAPTER TWO

CIRCATIDAL RHYTHMICITY IN METABOLIC AND IMMUNOLOGICAL INDICES OF ORGANISM CONDITION

CHAPTER TWO

CIRCATIDAL RHYTHMICITY IN METABOLIC AND IMMUNOLOGICAL INDICES OF ORGANISM CONDITION

2.1 INTRODUCTION.

As previously discussed in chapter one before any investigation of an index's response to a variety of imposed stresses can be seriously undertaken the background variability arising as a result of natural environmental phenomena, for example: tidal cycle, season, day length and life cycle/moult stage, must be elucidated.

For neritic marine invertebrates one of the most important facets of their ecology is the tidal nature of their environment and the regular changes in local conditions associated with water movement. Since the work of Naylor (1958) and Crothers (1968), first demonstrated endogenous circatidal rhythmicity in the locomotor activity of the common shore crab, *Carcinus maenas* (L.), with increased activity levels coincident with periods of high tidal elevation, many papers have highlighted the importance of tidal forcing on marine organisms (see review by Naylor, 1987). A large proportion of this type of research has been directed towards elucidating the nature of the endogenous clock or clocks involved in regulating locomotor activity (Reid and Naylor, 1989; Hunter and Naylor, 1993; and Reid and Naylor, 1993). By comparison, the influence of tidal forcing on invertebrate biochemistry has only received limited attention. Rajan *et al.* (1979), Williams (1985), and Delaney (1991) have all reported endogenous cyclical changes in the haemolymph sugar concentrations of *C. maenas,* with peak concentrations occurring at low water. Delaney (1991) has, in addition, reported a

similar rhythmicity in the concentration of haemolymph hydrogen peroxide, an end product of routine metabolism, which shows slight increases in concentration at low water.

The influence of tidal cycles on the internal "environment" of marine invertebrates has also been shown, particularly for molluscs, to include their immunocompetence. Most recently, Hawkins *et al.* (1993) have demonstrated tidally related changes in the immunocompetence of the American hard clam, *Mercenaria mercenaria.* Truscott and White (1991) first indicated a similar effect in crustaceans, in particular *C. maenas*, by demonstrating circatidal rhythms in total haemocyte count with maximum cell densities occurring during the high water period.

This chapter summarises an investigation into possible circatidal rhythmicity in a number of the biochemical and immunological parameters discussed in chapter one in an attempt to define the baselines from which further data could be assessed. More specifically this study has focused on the indices of haemolymph total sugar and hydrogen peroxide as well as the circulating haemocyte population and phenoloxidase enzyme activity.

To simplify the collection of data and to remove any other variables that would affect the indices the tidal entrainment of metabolic and immunological activity was assessed in a controlled laboratory environment (Lockwood *et al.*, 1982). With this design the influence of a regular cycling of water depth and hydrostatic pressure could be studied in isolation.

2.2 METHODS.

2.2.1 COLLECTION, MAINTENANCE AND GENERAL EXPERIMENTAL PROCEDURE.

Specimens of *C. maenas* were collected using traps baited with mackerel, plaice or mullet deployed sublittorally (approximately 1m. below chart datum, tidal range approximately 4m.) in the River Itchen within Southampton Water (Position: 50° 45.75'N, 1° 22.70'W; see figure 1.02)

For the experimental entrainments only intermoult (stage C_4; Drach, 1939) male crabs, > 55mm. carapace width, were chosen to avoid sex linked differences in metabolism (Williams, 1985; Truscott & White, 1990; and Delaney 1991). Only those individuals that were apparently healthy, having no missing limbs, carapace lesions or any gross indication of shell disease, were selected.

Prior to the period of entrainment all collected crabs were held in atidal conditions and continuous darkness in the aquarium for between nine and fourteen days. This allowed the organisms time to acclimate to the aquarium conditions of salinity 33.0 ± (SD) 0.2‰ , temperature 16.3 ± (SD) 2.2°C; and was a sufficient period of time to ensure that any tidal or diurnal field associated rhythms were lost (Williams, 1985; and Delaney, 1991).

During this entrainment study the experimental organisms were maintained in continuous darkness in a computer controlled "tidal tank" (Lockwood *et al.*, 1982) in which the water level varied sinusoidally with a period of 12 hours 25 minutes but out of phase with the tides in Southampton Water. Each individual was held within a Netlon™ box on the base of the tidal tank. The tank allowed for a vertical water movement of 0.90m. which is equivalent to a pressure change of 0.09 atmospheres, sufficient to entrain cycles of locomotor and metabolic activity in *C. maenas* (Naylor & Atkinson, 1972; Williams, 1985; and Delaney, 1991).

Two experimental protocols were set up: in the first system, designated tidal, animals were subjected to regular hydrostatic pressure changes as a consequence of a rise and fall in the water level of the tank. In this first system the crabs did not suffer aerial exposure during the low water period. The second

protocol, designated exposed tidal, was the same as the first except the animals were held on a platform above the base of the tank so that they were exposed to air for two hours around low water.

A third atidal protocol was adopted and acted as a control environment; in this instance however, because of the constraints of space, the animals were allowed to move freely throughout the tank. During the entrainments the crabs in all systems were fed chopped fish (mackerel, sand eels, plaice and mullet) every two days, but were starved for twenty-four hours prior to analysis.

After a period of nine days entrainment crabs were removed from each of the three systems at regular intervals over a tidal cycle (low water to low water) and a haemolymph sample (0.5-1.5ml.) was withdrawn from each individual via the unsclerotized membrane between the coxopodite and basiopodite of the first pereiopod, using a sterile syringe and a 25 gauge needle. After sampling the animals were returned to their entrainment system and allowed to recover for at least a week before being sampled again. During the experimental entrainments there were no recorded mortalities but occasionally ($< 2\%$) the extracted haemolymph had a milky white appearance, indicative of a gross bacterial infection (Johnson, 1983). These haemolymph samples were discarded and the affected crab removed from the experiment.

Each entrainment condition consisted of a minimum of six animals and the experiments were repeated on different batches (a minimum of two) of fresh animals to increase the size of the data set collected. As a consequence of the limited amount of haemolymph available from each crab the metabolic indices of haemolymph sugar and hydrogen peroxide were measured initially and once completed the indices of immunocompetence (circulating haemocyte population and phenoloxidase enzyme activity) were assayed . At the end of the investigation all of the animals were released into the River Hamble (figure 1.02) - approximately twelve miles from their point of collection.

2.2.2 METABOLIC AND IMMUNOLOGICAL METHODS.

Measurements of total sugar and hydrogen peroxide concentration were made at the same time on each of the haemolymph samples collected. Before the collected haemolymph samples were deproteinated for the sugar assay 0.1ml. aliquots were removed for the hydrogen peroxide determination. The haemocyte counts and phenoloxidase enzyme activities were made using separate samples however, as the haemocytes had to be fixed in formaldehyde before they could be counted (section 2.2.2.3).

2.2.2.1 HAEMOLYMPH SUGAR DETERMINATION.

The concentration of anthrone responsive sugars (total reducing sugars, the mono- and oligosaccharides) was determined using the method described by Roe (1955).

Haemolymph samples (between 0.5 and 1.5ml.) were removed from two crabs at each sampling interval and diluted with a deproteinising reagent (5% v/v trichloroacetic acid) to approximately 1/10 of their original concentration. The mixtures were shaken and allowed to stand for five minutes after which time the samples were centrifuged at 2000rpm., equivalent to 450*g*, for fifteen minutes. 1ml. of the supernatant was placed in a boiling tube to which 10ml. of anthrone reagent was added. Similar boiling tubes were prepared containing benzoic acid as a blank; and containing 0.1mg. per ml. glucose solution, as a standard. All of the tubes were placed in an enclosed diethylene glycol bath at 100°C (Cooper and McDaniel, 1970) for 15 minutes, chilled in iced water and held in the dark for thirty minutes. Once cool the absorbances of the samples and standards were compared, relative to the blank, using a Cecil CE 292 Digital Ultraviolet Spectrophotometer, set to a wavelength of 620nm.

Having confirmed agreement with the Lambert & Beer law (Roe, 1955) over the range of expected blood sugar levels, the concentration of sugars (in mg. per one hundred ml.) was determined using the following equation (Roe, 1955):

$$[SUGAR]\ (mg.100ml^{-1}) = \frac{DU}{DS} \times 0.1 \times BLOOD\ DILUTION \times 100 \quad \textbf{(1)}$$

DU = absorbance of sample.

DS = absorbance of standard.

0.1 = mg. of glucose in 1ml. of standard solution.

2.2.2.2 HYDROGEN PEROXIDE DETERMINATION.

The hydrogen peroxide concentration within the haemolymph samples was determined using the method of Meiattini (1984) which uses a hydrogen donor (D) (4-aminophenazone/ chromotropic acid system) that is colourless in the reduced form and blue when oxidised. From the stoichiometry of the principal equation given below:

$$H_2O_2 + DH_2 \xrightarrow[EC.1.11.1.7.]{Hydrogen\ Peroxide\ Oxidoreductase} 2H_2O + D \quad \textbf{(2)}$$

- it can be seen that the intensity of the blue coloration produced in this assay is proportional to the initial hydrogen peroxide concentration.

0.1ml. haemolymph samples were removed from the crabs at each sampling interval. Hydrogen peroxide is a labile compound quickly disassociating into water and toxic oxygen radicals, and is subsequently rapidly broken down by enzyme systems within the organism. To counter this problem as soon as the samples were collected they were immediately treated with a deproteinising agent (0.1ml. of M perchloric acid solution) after which they were considered to be stable for 18 hours (Meiattini, 1984).

Following addition of the perchloric acid the samples were centrifuged at 3000rpm., equivalent to 750*g*, for ten minutes before being neutralised with 0.01ml. of 6M sodium hydroxide solution. 0.1ml. of this final solution was used for analysis, being mixed with 2.5ml. of peroxide reagent (warmed to room

temperature, see Meiattini, 1984) before being stirred and allowed to stand for five minutes. Similar additions of peroxide reagent were made to 0.1ml. deionised water (blank) and to two aliquots of 0.1ml. hydrogen peroxide standard (20mg. H_2O_2 per litre).

After the five minute incubation the absorbances of the samples and standards were compared relative to the blank, using a spectrophotometer set at a wavelength of 600nm.

After determination of agreement with the Lambert & Beer law over the expected concentration range the hydrogen peroxide concentration was calculated using the following equation:

$$[H_2O_2]\ (mg\ l^{-1}) = \frac{DU}{DS} \times 20 \times 2.1 \qquad (3)$$

- where 2.1 is a correction factor applied to account for the initial dilution of the haemolymph sample (0.1ml. of haemolymph in a total volume of 0.21ml.).

2.2.2.3 TOTAL AND DIFFERENTIAL HAEMOCYTE COUNTS.

Total haemocyte counts were made at each sampling interval using an "Improved Neubauer" pattern haemocytometer viewed with Nomarski contrast interference optics fitted to an Olympus™ BH2 microscope. To avoid problems of haemolymph clotting during the extraction procedure or during subsequent counting a quantitative volume of haemolymph was withdrawn from each crab into a syringe containing 0.5ml. of 20% seawater formaldehyde solution as a fixative (Truscott & White, 1990).

Differential counts were initially inferred from blood smears stained with the fat stain Sudan Black B according to the method of Sheehan (1939) (quoted in Gray, 1954). Using this method hyalinocytes (size 6-10μm.) were distinguished as staining entirely black (Hose *et al.*, 1987; Hose *et al.*, 1990) whilst granulocytes (10-15μm.) stained faint blue as shown in plate 2.01. Eventually quantitative

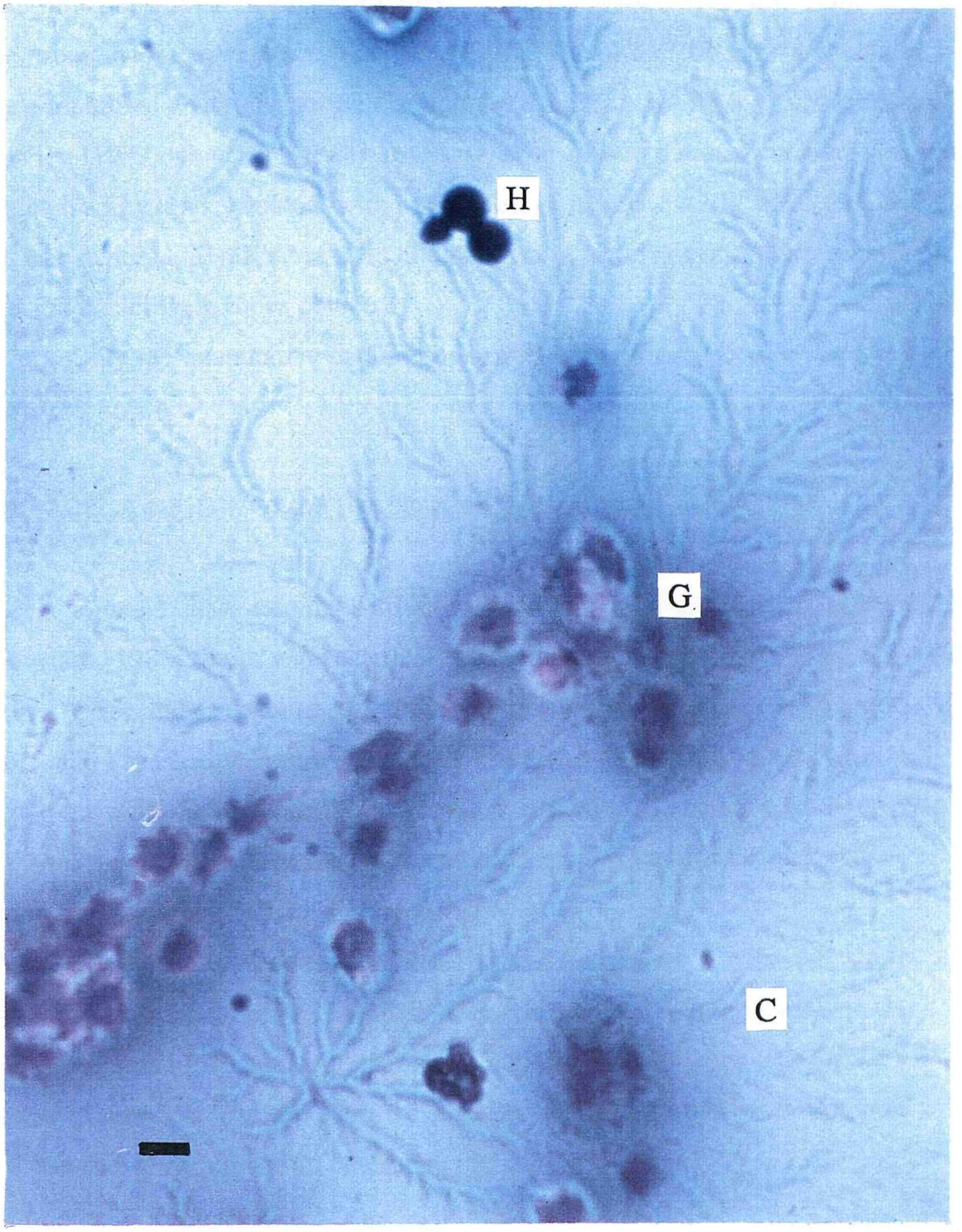

PLATE 2.01 *Carcinus maenas* haemolymph smear stained with Sudan Black B and Methylene Blue according to Sheehan (1939). H = hyalinocytes stained exclusively with Sudan Black B (Hose et al., 1990). G = clotted granulocytes stained with Methylene Blue showing purple nuclear material surrounded by a lighter cytoplasm. C = crystals formed where the haemolymph smear was too thick and dried too slowly. Scale bar = 10μm.

determinations of the number of granulocytes and hyalinocytes were made using the haemocytometer chamber (see plate 2.02). The different haemocyte classes were identified according to their morphology and size according to Ratcliffe and Rowley (1979), Bauchau (1981) and Hose *et al.* (1990). Granulocytes tended to be larger than hyalinocytes and were packed full of granules which often obscured the nuclear material. Hyalinocytes, in contrast, had a less densely packed cytoplasm and the nuclei were more obvious.

Only the quantitative data are presented in the results section (see section 2.3).

2.2.2.4 PHENOLOXIDASE ENZYME ASSAY.

Phenoloxidase enzyme activity was determined by adapting the methods of Söderhäll, 1981; Smith and Söderhäll, 1983; Söderhäll and Smith, 1983; Leonard *et al.*, 1985; and Jackson *et al.*, 1993. For this assay all glassware and pipette tips were acid - washed to remove any trace oxidants before being rinsed twice in deionised water and once in Milli-Q™ water and sterilised by autoclaving at 120°C for twenty minutes to remove any bacterial films. All buffers and reagents were made up with Milli-Q™ water as required.

The phenoloxidase enzyme assay was performed in two separate stages discussed separately below. The first stage (haemocyte lysate preparation) was used to separate the haemocytes from the collected haemolymph and isolate the zymogen prophenoloxidase from the granulocytes. The second stage was used to activate the zymogen and to determine the enzyme activity.

Haemocyte Lysate (HLS) Preparation.

0.6ml. of haemolymph was separately withdrawn from each crab into a sterile syringe which contained 0.4ml. of ice cold citrate EDTA buffer (pH = 4.6) (Söderhäll and Smith, 1983) as an anticoagulant. For this assay the samples from two crabs were pooled immediately after collection to obtain a sufficient haemolymph volume to produce a useable haemocyte pellet.

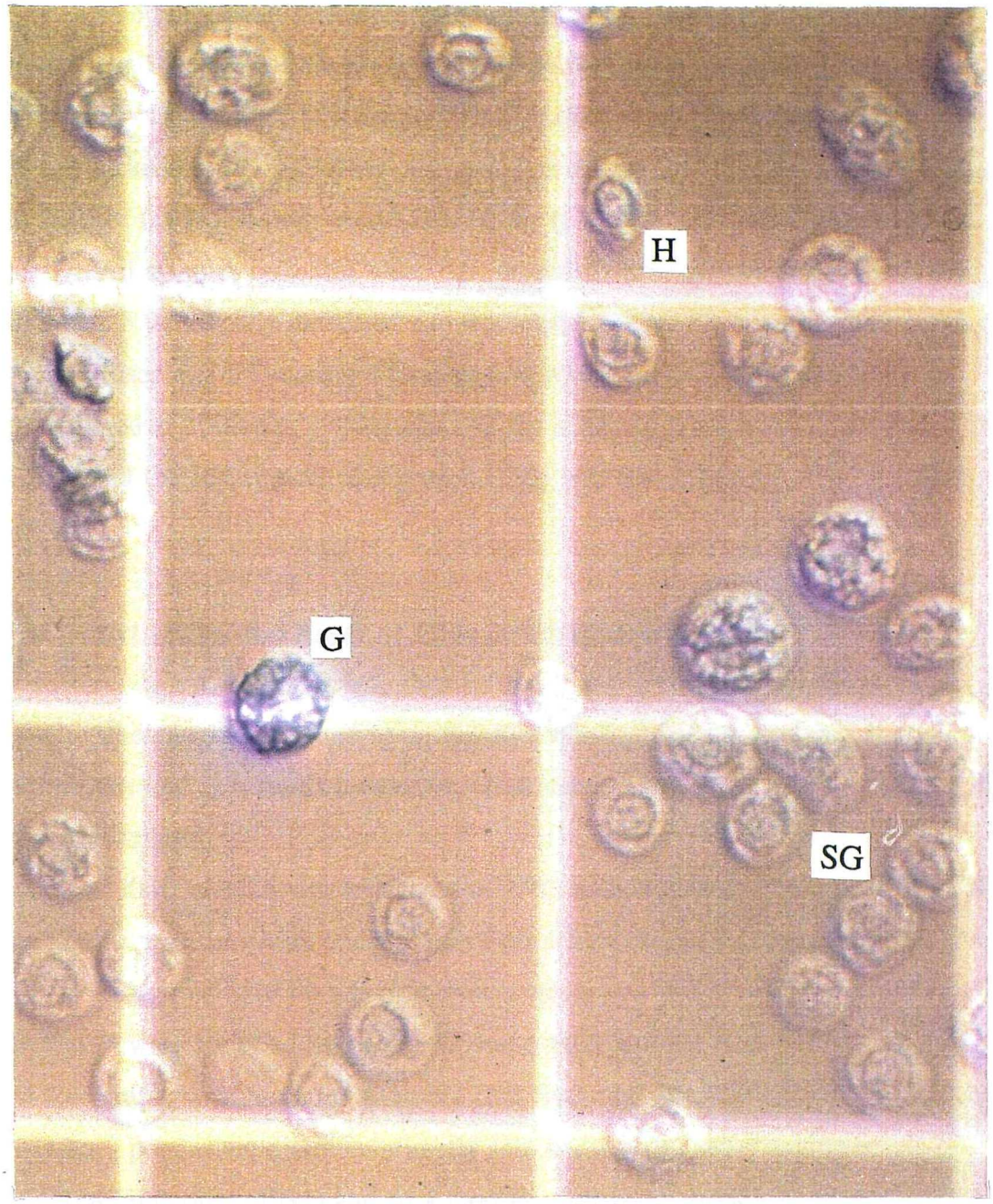

PLATE 2.02 Haemocytometer counting chamber showing a 20% seawater formaldehyde fixed haemolymph sample viewed with Nomarski contrast interference optics (x 400). G = Granular haemocytes showing cytoplasm packed with granular material occluding the nucleus. SG = Semigranular haemocytes showing a less densely packed cytoplasm and more visible nucleus. H = Phagocytic hyalinocyte, smaller in size than the granulocytes and with a clearly visible nucleus. Grid represents 50x50μm.

The pooled samples were then centrifuged at 2000rpm. (450*g*) for 15 minutes and the pellet of haemocytes was washed twice, without resuspension, with 2ml. of a solution of ice cold 0.01M sodium cacodylate citrate buffer (pH = 7; see Jackson *et al.*, 1993) before rapid freezing by immersion in liquid nitrogen, in 2ml. of 0.01M sodium cacodylate buffer (pH = 7; see Jackson *et al*, 1993).

All samples collected were then stored frozen until the time of analysis when each was slowly defrosted and homogenised, on ice, using a glass piston homogeniser in a 5°C constant temperature room. Each sample was then centrifuged at 2000rpm. (900*g*) and 3°C for a further 25 minutes to remove cell debris and the supernatant, designated HLS, was then assayed for phenoloxidase activity.

Activation and Assay of Phenoloxidase Activity.

Phenoloxidase occurs as an inactive zymogen within the granulocytes of arthropods and as a result the enzyme first needed activating with an elicitor, in this case trypsin.

400μl. of HLS was incubated at 15°C with 400μl. of a solution of 0.1% trypsin (0.5 Anson Units per gramme from beef pancreas) in cacodylate buffer.

After one hour 400μl. of dihydroxyphenylalanine (L-dopa, 4g. per litre in Milli-Q™ water) was added to the activated HLS and the change in the absorbance of the sample was measured over the first five minutes. The stability of the L-dopa was very poor when dissolved directly into cacodylate buffer (as described in Smith & Söderhäll, 1991) and so a modification was introduced and the solution was prepared with Milli-Q™ water.

To take account of any background oxidation of the L-dopa as a consequence of the assay procedure or the inactive HLS a control was prepared for each individual sample. The controls, consisting of 400μl. of HLS, 400μl. of cacodylate buffer (to replace the trypsin) and 400μl. of L-dopa, were treated in an identical manner to the samples. To account for any oxidation of L-dopa by the reagents used reagent blanks were prepared in which the HLS was replaced with an equal volume of cacodylate buffer.

Enzyme activity was expressed in arbitrary units where one unit represents an increase in absorbance of the sample of 0.001 min^{-1} at 490nm. (Söderhäll & Smith, 1983), all measurements being made on a spectrophotometer.

The protein content of the HLS was determined using the procedure described by Raymont *et al.* (1964) which makes use of the biuret reagent. For the determination 2ml. of biuret reagent were added to 0.5ml. of HLS and incubated for thirty minutes at room temperature before being centrifuged at 2000rpm. (450*g*) for five minutes. The optical density of the supernatant was compared with a bovine serum albumen standard (fraction V, 10mg. protein per ml.) using a spectrophotometer set at a wavelength of 540nm.

2.2.3 MEASUREMENT OF CARDIAC ACTIVITY.

From the data collected relating to changes in the concentration of haemolymph sugar and hydrogen peroxide as well as the haemocyte population of the *tidally exposed* crabs predictable cycles associated with the tidal regime and routine air exposure were observed as have been discussed below. In an attempt to explain these observed cycles it was hypothesised that changes in the cardiac activity of the exposed animals may have resulted in the measured fluxes. To this end an investigation was made of the heart activity of crabs in all three entrainment systems to verify the proposed hypothesis.

After a period of development heart rate was finally measured using a modification of the method described by Helm and Trueman (1967) who used an impedance coupler to measure cardiac activity and small valve movements in the mussel, *Mytilus edulis.*

Crabs were anaesthetised with a 0.5ml. solution of 0.5% procaine and two varnished copper wires, with the terminal 5mm. bared, were inserted through holes drilled in the dorsal carapace either side of the heart at an angle of approximately 45° from the mid line and cemented in place with epoxy resin. Following the operation the crabs were allowed a period of twenty-four to forty - eight hours to recover before any recordings were made.The electrodes were connected via a multiplexing board (PC Lab Card™ "Relay Multiplexer Board

Model PCLD-788"), which allowed six crabs' hearts to be monitored sequentially, to an impedance unit (Strathkelvin Instruments, Glasgow) integrated with a George Washington™ 400/MD2 Bioscience Recorder. The impedance coupler generated an alternating current (30kHz, 8mA rms.) which was passed between the electrodes; changes in heart volume created fluctuations in impedance between the electrodes and these fluxes were recorded initially as a paper trace.

In order to cope with the large volume of data that was generated, the voltage output from the George Washington™ recorder was logged on a 386 DX40 personal computer via an analogue to digital converter (PC Lab Card™ "Multi Lab Card with Programmable Gain, PCL-812 PG") controlled using Advantech Genie™ "Data Aquisition and Control Software" (Version 1.0B). Figure 2.01 indicates a block diagram showing the layout of the system.

Recordings of cardiac activity were analysed using Fourier analysis. The stored heart trace data was downloaded into Mathcad™ 4.0 software (see figure 2.02) and was transformed using a complex Fourier transform to produce a power curve as shown in figure 2.03. The power curve was used to indicate the dominant sine wave component (wave number) of the signal, the frequency of which could be determined using the relationship described in equation 4:-

$$f_j = \frac{j}{n} \times f_s \qquad (4)$$

j = wave number.

f_j = frequency of wave number j.

f_s = sampling frequency (25Hz).

n = number of scans in heart trace data set.

Depledge (1978) has indicated that the magnitude of the output voltage from the impedance coupler can be used to indicate the size of the cardiac contraction, and as a result the root mean square output voltage (rms.) was also determined from the collected data to give an indication of the size of the heart

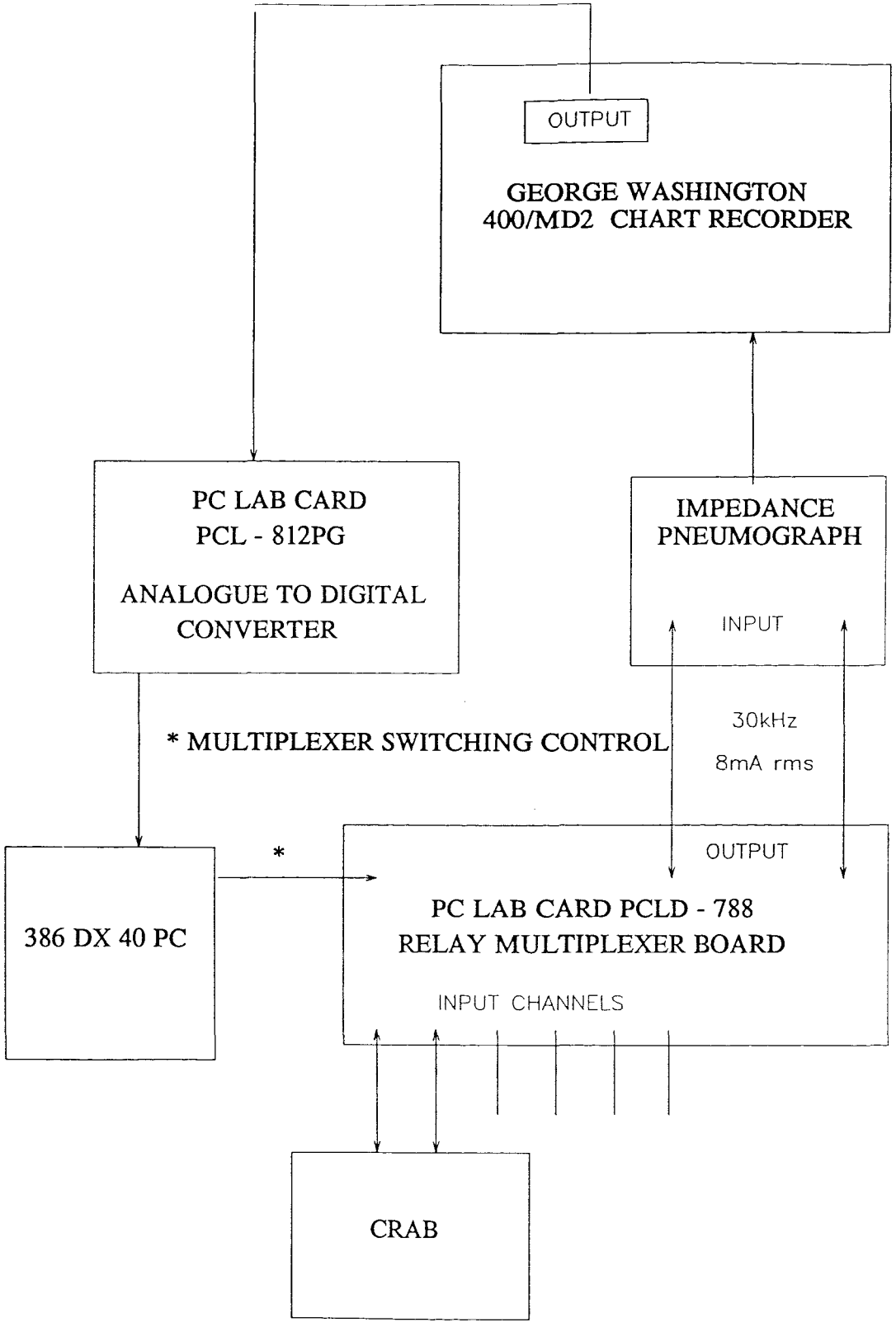

FIGURE 2.01 Block diagram summarising the layout of the apparatus used to measure cardiac activity.

beats.

The final modified method was used following a period of development. Initial studies attempted to measure direct cardiac voltages. Crabs were anaesthetised with a 0.5ml. solution of 5% procaine and two copper wires were inserted through drilled holes in the dorsal carapace either side of the heart and cemented in place with cryanolate adhesive (Bostik Super Glue™). The output of the electrodes was connected to a Servoscribe 1S chart recorder. Traces of voltage output were recorded under a variety of conditions (for example emersion, immersion and during periods of locomotor activity) and whilst periods of cardiac output were recorded, the signal was often swamped with electrical noise from nearby muscle tissue and surrounding appliances.

As a consequence and following consultation with another worker in the field (A.C. Taylor (pers. comm.)) the impedance technique used by Helm and Trueman (1967) on the mussel, *Mytlius edulis*, was adapted. This method required that the electrodes be connected to an impedance unit.

Although initial results were promising (the first organism tried produced strong heart traces after 96 hours of recovery) problems were encountered with the fixation of electrodes to the carapace, with generating reliable and reproducible heart traces and with the long term melanisation of the implanted electrode tips involving the phenoloxidase cascade, so another method (Dyer & Uglow, 1977) was attempted. This method initially appeared more attractive as it required that only one electrode was inserted through the carapace, the common electrode being an aluminium sheet placed in the seawater medium, and was therefore less restrictive. Tests with an artificial organism (a block of polyacrylamide gel) demonstrated that this method also responded to relative movement between the "organism" and the aluminium common electrode and as a result proved unsuitable.

Further discussion (Hauton D., pers. comm.) revealed that the anaesthetic procaine suppresses heart activity and that the dose used was much greater than required. Reverting back to Helm & Trueman's (1967) method and using a ten - fold dilution of the procaine produced a more rapid recovery of the crab after the operation and did not suppress cardiac output. At the same time epoxy resin

(Araldite™ Rapid Adhesive) was tested as an alternative to cryanolate adhesive and was found to be superior.

2.2.4 STATISTICAL ANALYSIS OF DATA.

A number of statistical tests were used to indicate significant differences between the three entrainment conditions (tidal, exposed tidal, and atidal/control) and also differences between the high and low water states for individual entrainment conditions.

All of the data were initially tested for normality and in those cases where the data had a parametric distribution the comparisons between the entrainment conditions were made using one way analysis of variance (ANOVA) to indicate significant differences in the variances of the data sets. In those instances where the variances were significantly different Dunnetts' tests were performed to indicate the magnitude and direction of the differences in the data. For those occasions when the data were not normally distributed the less stringent Kruskal-Wallis ANOVA and subsequent Dunn's test were used to compare entrainment conditions.

Comparisons of between high and low water values for each entrainment condition were made using *t* tests in the case of parametrically distributed data and Mann-Whitney Rank Sum tests when the data were not normally distributed.

Significant differences were observed for all comparisons when $P < 0.05$.

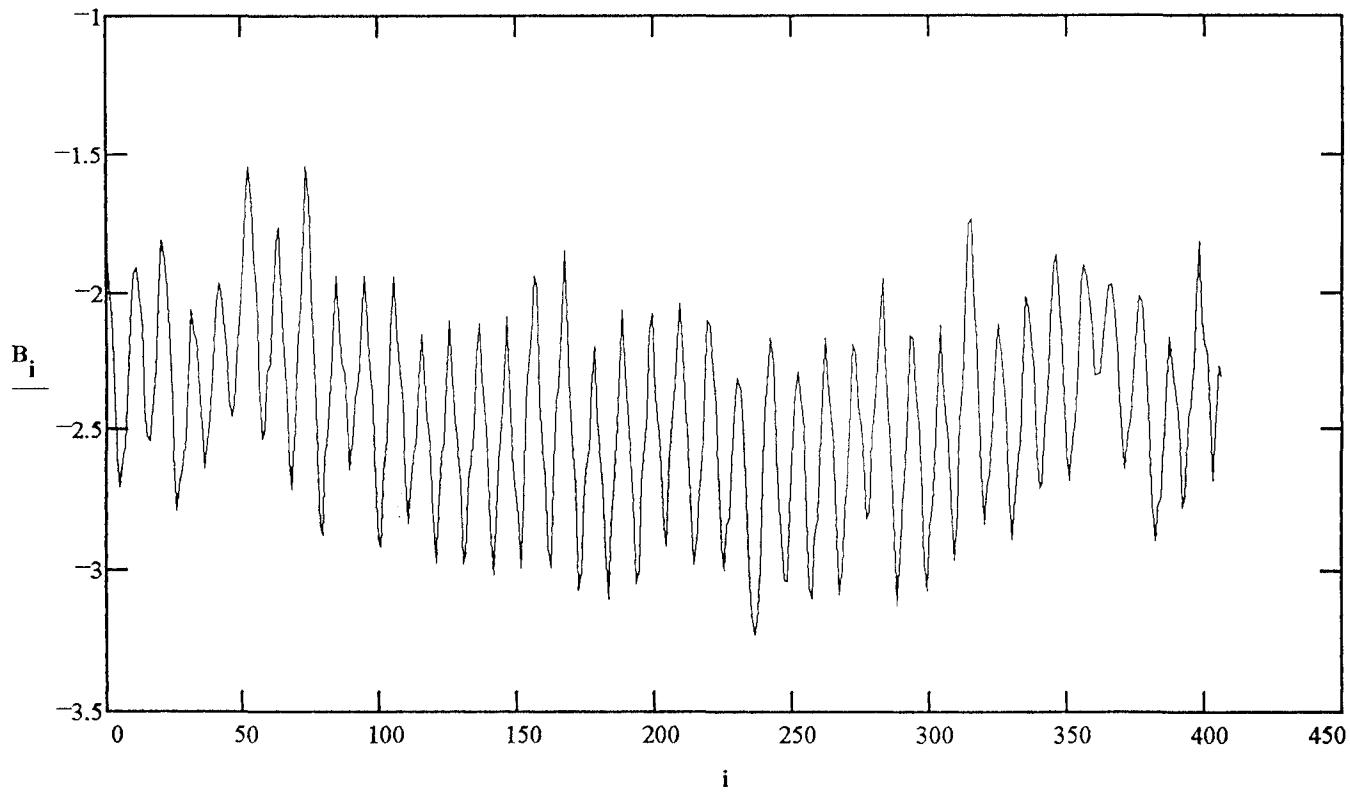

FIGURE 2.02 Typical heart record from a single specimen. The abscissa (i) represents scan number (taken from the multiplexer board; twenty-five scans approximates one second) and the ordinate (B_i) represents the output voltage (volts) from the impedance coupler.

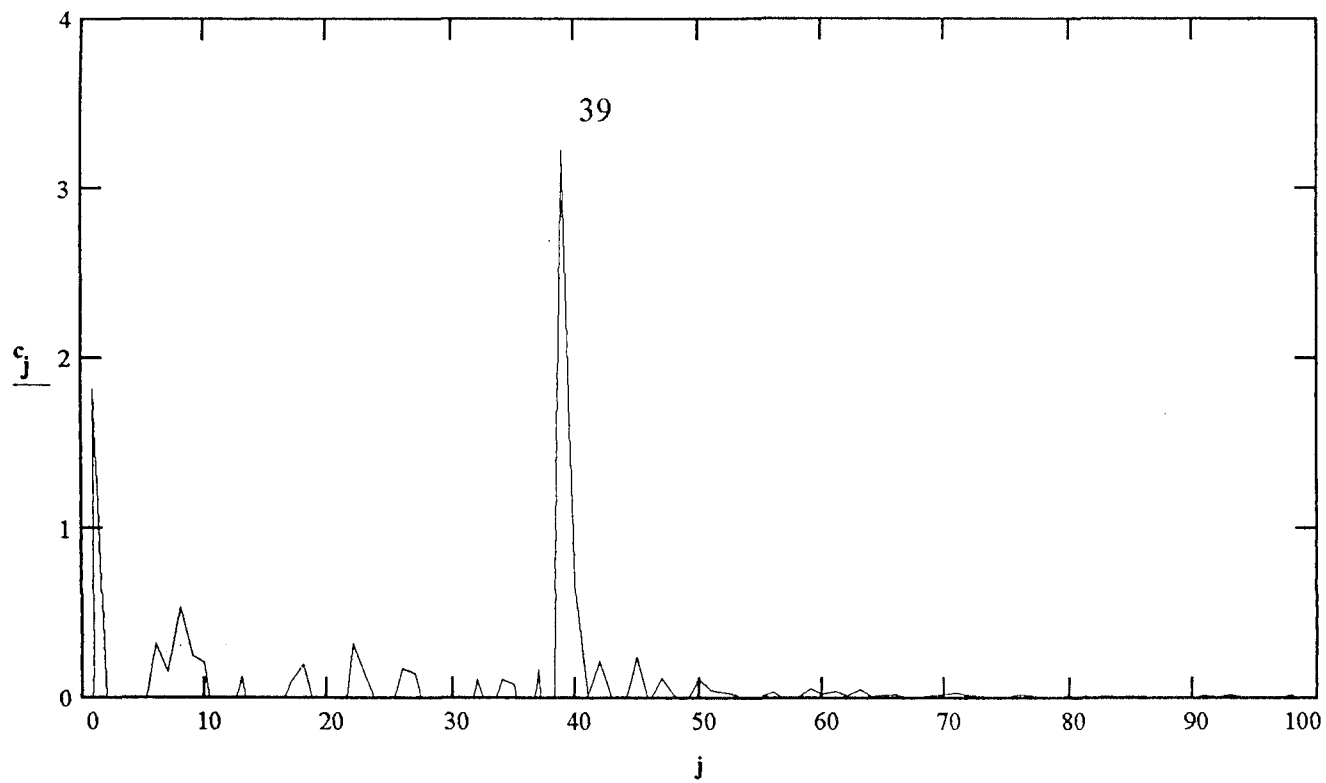

FIGURE 2.03 Power curve generated from a complex Fourier transform of the signal shown in figure 2.02. The numbered peak represents the dominant "wave number", the frequency of which can be determined from equation 4. The dominant wave number represents the cardiac contractions, the remaining smaller peaks represent the different frequencies of electrical and other noise.

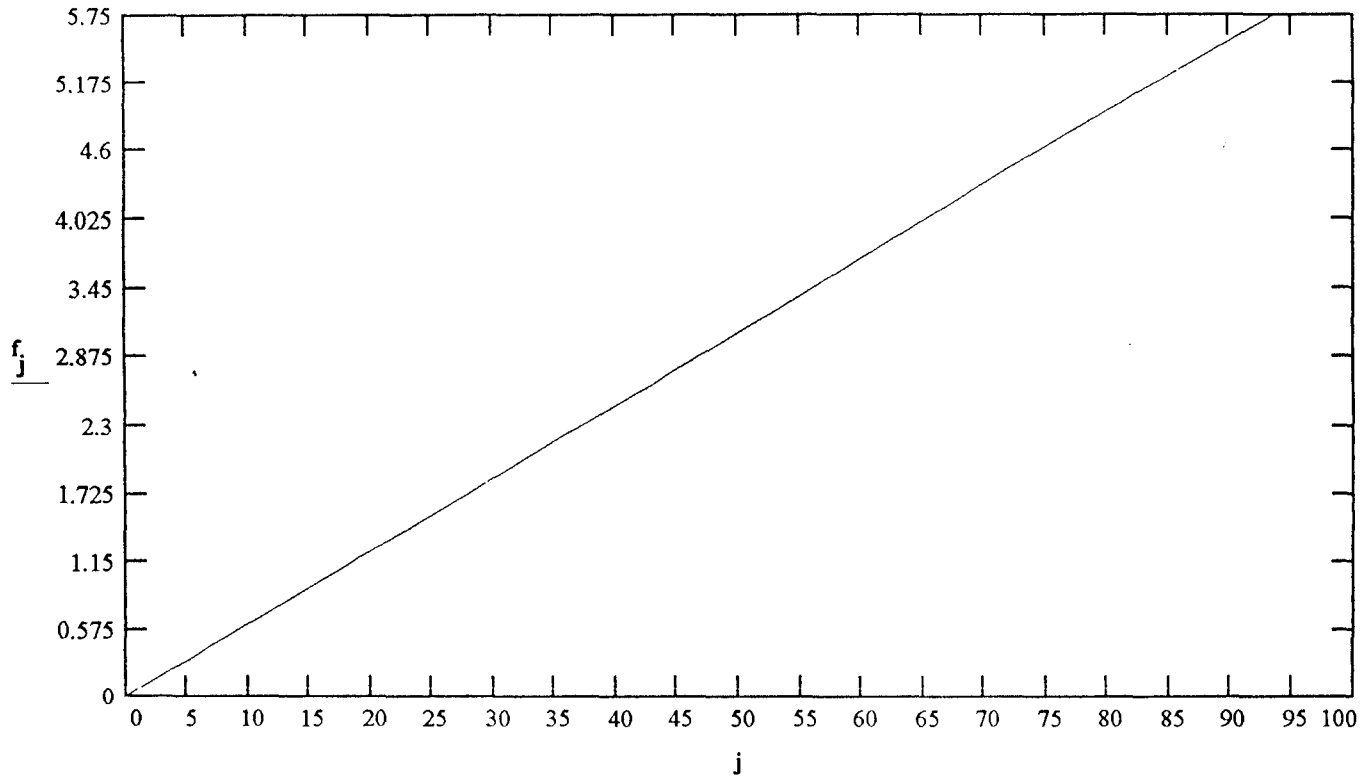

FIGURE 2.04 Graphical representation of equation 4 using the heart trace shown in figure 2.02 showing wave numbers (j) along the abscissa and frequency (f_j, Hz) along the ordinate.

2.3 RESULTS

2.3.1 BIOCHEMICAL AND IMMUNOLOGICAL DATA.

2.3.1.1 HAEMOLYMPH SUGAR CONCENTRATION.

Fluctuations in the haemolymph anthrone - responsive sugar concentrations have been summarised in table 2.01 and in figure 2.05 from which it can be seen that the mean sugar concentration in the haemolymph of the tidally entrained crabs peaked at low water (means: 48.29 $\pm$ (SD) 42.26 and 56.65 $\pm$ 32.29 mg. per one hundred ml.) and reached a minimum of 20.24 $\pm$ 9.33 mg. per one hundred ml. at high water.

For the tidally exposed organisms haemolymph sugar peaked at high water (76.00 $\pm$ 28.37 mg. per one hundred ml.) and reached its lowest concentration at low water (51.85 $\pm$ 23.24 and 50.91 $\pm$ 9.44 mg. per one hundred ml. respectively). In the control environment haemolymph sugar concentrations remained constant at approximately 48 mg. per hundred ml.

Normality tests were carried out and, where possible, variation in haemolymph sugar within a single entrainment condition was assessed using *t* tests to compare the concentrations at high and low water. In the cases where the data was not normally distributed and parametric tests were not possible, the less stringent Mann-Whitney Rank Sum test was used. In all cases however no significant differences were found between the haemolymph sugar concentration at low water and high water for any entrainment condition.

One way analysis of variance (ANOVA) tests were used to determine whether there was any significant difference in blood sugar concentration across the three entrainment conditions at the two low waters and high water. There was a significant (Dunnett's test, $P < 0.05$) elevation of blood sugar in the tidally exposed individuals when compared to the control organisms at high water only.

Figure 2.06 shows the first order linear regression relationships between haemolymph sugar and tidal elevation for the tidally exposed and entrained individuals. It can be seen that there is a significant negative correlation between

TIDAL STATE	EXPOSURE CONDITION					
	TIDAL		EXPOSED TIDAL		ATIDAL/CONTROL	
	[BLOOD SUGAR] (mg. $100ml^{-1}$).	N	[BLOOD SUGAR] (mg. $100ml^{-1}$).	N	[BLOOD SUGAR] (mg. $100ml^{-1}$).	N
LOW WATER	48.29 ± 42.26	13	51.85 ± 23.24	9	49.44 ± 43.11	13
LW + 4	35.18 ± 19.57	11	73.58 ± 19.78	9	48.72 ± 76.22	10
HIGH WATER	20.24 ± 9.33	12	76.00 ± 28.37	9	38.35 ± 14.66	13
LW + 8	33.23 ± 30.28	12	66.64 ± 29.23	6	49.66 ± 53.80	11
LOW WATER	56.63 ± 32.29	10	50.91 ± 9.44	6	54.88 ± 33.61	10

TABLE 2.01 Variation in anthrone responsive sugar concentration with exposure condition and tidal state. All values represent the mean ± one standard deviation of N observations (one observation per crab), and the atidal series represents the control environment.

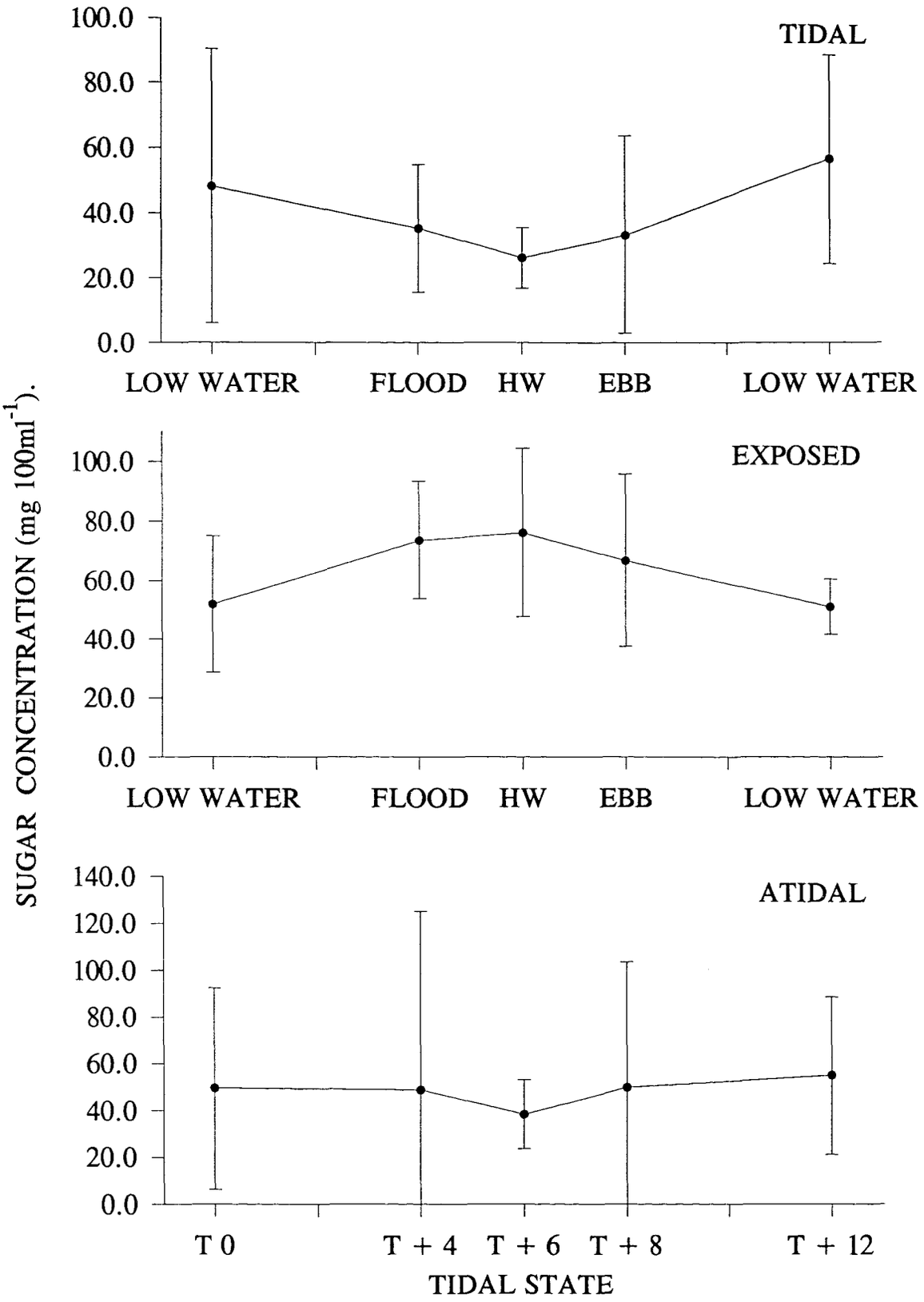

FIGURE 2.05 Variation in haemolymph total sugar concentration with respect to tidal state and entrainment condition showing the mean ± one standard deviation of the collected data as summarised in table 2.01.

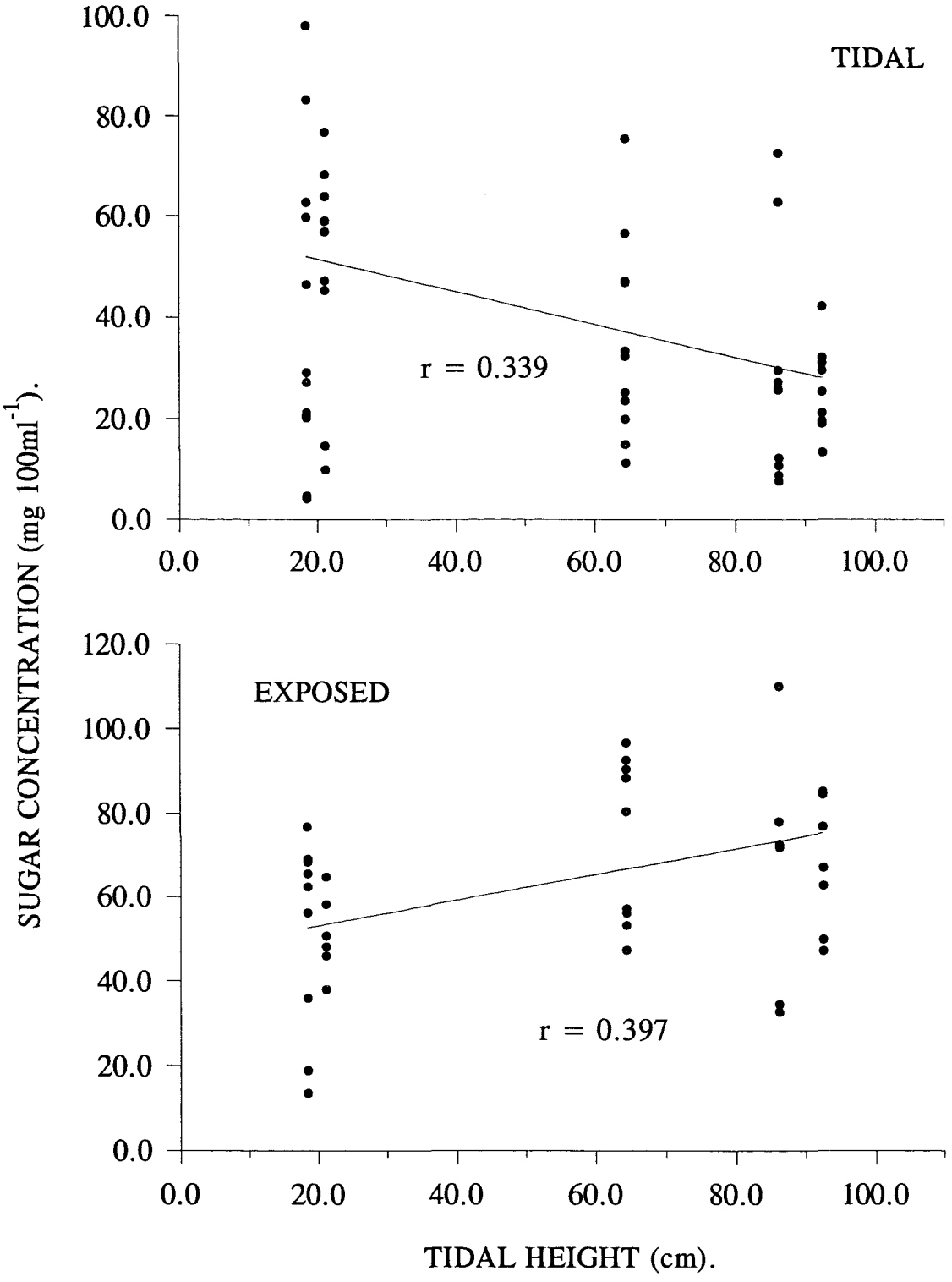

FIGURE 2.06 Regression relationships between tidal elevation and haemolymph total sugar for the tidal and exposed crabs. Figure shows the regression relation for all of the collected data.

tidal elevation and haemolymph sugar ($r = 0.339$, $P < 0.05$) for the tidally entrained organisms whilst the relationship is positive in the case of the tidally exposed crabs ($r = 0.397$, $P < 0.05$).

2.3.1.2 HAEMOLYMPH HYDROGEN PEROXIDE CONCENTRATION.

Changes in hydrogen peroxide concentration associated with tidal state and entrainment condition have been summarised in table 2.02 and figure 2.07 from which it can be seen that there was a general rising trend in haemolymph hydrogen peroxide with tidal elevation for the tidally entrained crabs although the high water value was anomalous. Hydrogen peroxide concentrations varied between 21.92 ± 10.63 and 32.90 ± 18.91 mg. per litre for the tidally entrained crabs.

For the exposed animals changes in hydrogen peroxide were more consistent firstly decreasing towards high water reaching a minimum of 12.69 ± 13.10 mg. per litre before rising again during the ebb to low water peaking at 25.52 ± 11.75 mg. per litre.

Hydrogen peroxide concentrations in the atidal organisms fluctuated between a minimum of 13.42 ± 6.75 and 32.27 ± 22.91 mg. per litre with no obvious trends.

Figure 2.08 shows the linear regression relations between hydrogen peroxide concentration and tidal elevation for the tidally entrained and exposed crabs. As with haemolymph sugar concentration there was a reversal of the general relationship between the tidally entrained and the exposed crabs. For the tidally exposed organisms there was a significant negative correlation ($r = 0.380$, $P < 0.05$) between hydrogen peroxide and tidal height whilst the correlation was positive for the tidally entrained crabs ($r = 0.211$, $P > 0.05$).

As was found with haemolymph sugar concentrations, statistical tests failed to show any significant difference between high and low water hydrogen peroxide concentrations within each entrainment condition. Kruskal-Wallis analysis of variance and a subsequent Dunn's test demonstrated a significantly higher ($P < 0.05$) haemolymph hydrogen peroxide concentration at low water in the exposed

TIDAL STATE	EXPOSURE CONDITION					
	TIDAL		EXPOSED TIDAL		ATIDAL/CONTROL	
	[BLOOD HYDROGEN PEROXIDE] (mg. l^{-1}).	N	[BLOOD HYDROGEN PEROXIDE] (mg. l^{-1}).	N	[BLOOD HYDROGEN PEROXIDE] (mg. l^{-1}).	N
LOW WATER	22.15 ± 6.92	14	24.05 ± 14.81	12	13.42 ± 6.75	14
LW + 4	32.90 ± 18.91	11	16.56 ± 11.31	10	25.82 ± 18.15	13
HIGH WATER	23.09 ± 13.87	10	12.69 ± 13.10	11	18.07 ± 9.19	19
LW + 8	32.48 ± 18.15	12	13.02 ± 7.27	10	32.27 ± 22.91	10
LOW WATER	21.92 ± 10.63	12	25.52 ± 11.75	10	17.48 ± 13.35	14

TABLE 2.02 Variation in haemolymph hydrogen peroxide concentration with exposure condition and tidal state. All values represent the mean ± one standard deviation of N observations (one observation per crab, two experimental batches), and the atidal series represents the control environment.

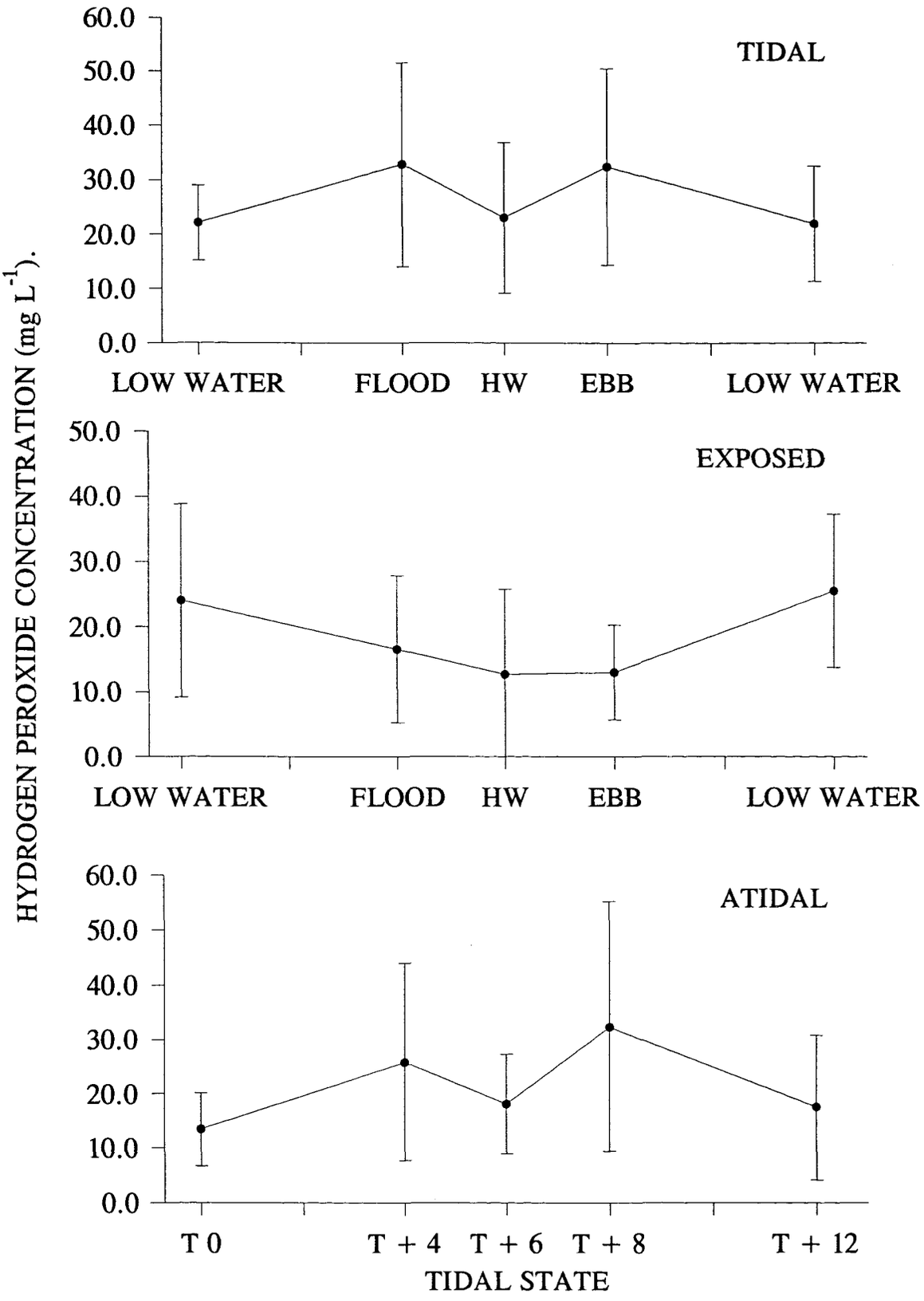

FIGURE 2.07 Variation in haemolymph hydrogen peroxide with respect to tidal state and entrainment condition showing the means and standard deviations summarised in table 2.02.

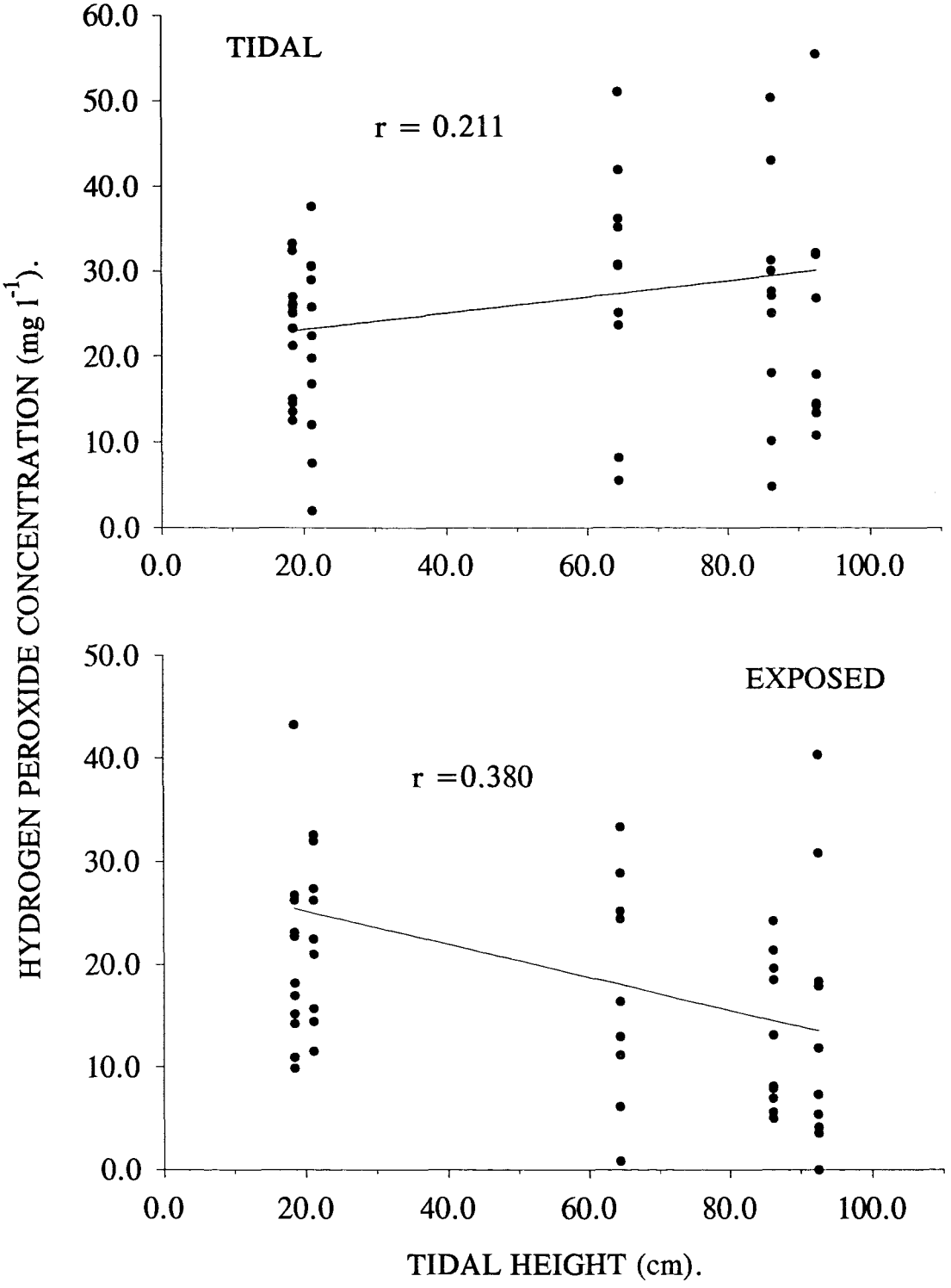

FIGURE 2.08 Linear regression relationships between tidal height and haemolymph hydrogen peroxide for the tidal and exposed crabs. Figure shows the complete data set rather than the means only.

individuals when compared to the control atidal environment, all other comparisons proving non significant.

It can be hypothesised that the utilisation of glucose and the production of hydrogen peroxide through routine metabolism are intrinsically linked. To this end the sugar and hydrogen peroxide data have been plotted together for all three entrainment conditions as shown in figure 2.09 which indicates that there is a significant negative correlation between the concentration of haemolymph sugars and hydrogen peroxide (Pearson Product Moment Correlation Coefficient = -0.597, $P < 0.05$).

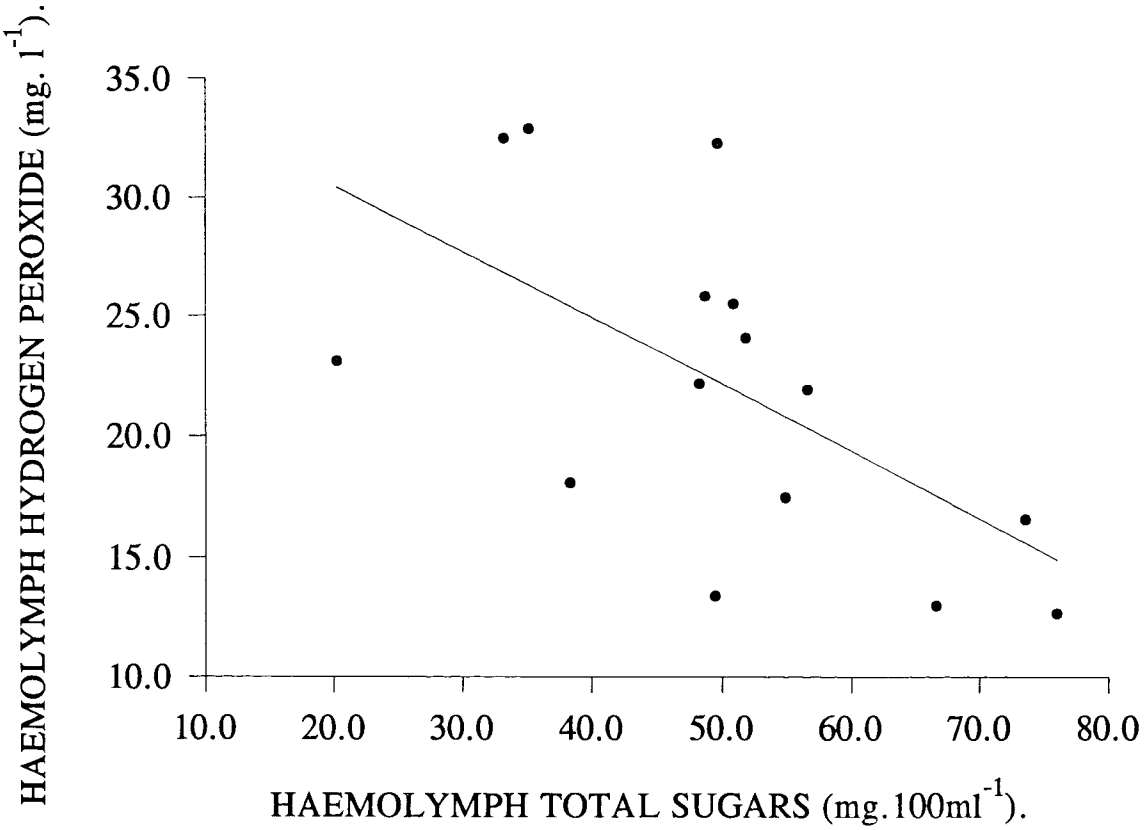

FIGURE 2.09 Pearson product moment correlation between haemolymph total sugar and hydrogen peroxide concentrations. See text for explanation.

2.3.1.3 TOTAL AND DIFFERENTIAL HAEMOCYTE COUNTS.

Total and differential haemocyte counts have been summarised in table 2.03 which shows the mean, standard deviations and sample sizes. Figures 2.10 and 2.11 present the data graphically as total haemocyte count (THC) and as the ratio of granulocytes to hyalinocytes respectively. From figure 2.10 it can be seen that the haemocyte population in all exposure conditions fluctuated around 30 x 10^6 cells per ml.

For the tidally entrained animals the mean THC peaked during the flood (34.10 $\pm$ 15.06 x 10^6 cells per ml.) and reached its minimum during the ebb (22.98 $\pm$ 10.56 x 10^6 cells per ml.) The haemocyte population in those crabs entrained to the exposed tidal system peaked during low water (35.10 $\pm$ 20.26 and 39.24 $\pm$ 7.58 x 10^6 cells per ml. respectively) and tended to decrease approaching and during high water. The cell population in the atidal system fluctuated between a maximum of 39.63 $\pm$ 23.25 x 10^6 and 25.45 $\pm$ 11.00 x 10^6 cells per ml. with no obvious trends.

Figure 2.11 shows that for all entrainment conditions the ratio of granulocytes to hyalinocytes fluctuated around 3:1. There was little variation in this ratio, and no consistent pattern with respect to time or tidal elevation.

Normality tests were carried out on all data and, where possible, single factor ANOVA was performed to determine whether there were any significant differences between the three entrainment conditions at the two low waters and high water. Where the data was not normally distributed and parametric comparisons were not possible, the less stringent Kruskal-Wallis test was performed. In all cases however no significant differences were found in the THC or the granulocyte: hyalinocyte ratio at any tidal state tested.

To compare variation within a single entrainment condition the haemocyte populations at low and high water were compared using either *t* tests and Mann-Whitney Rank Sum tests (used for parametric and non parametric data respectively). As before no significant differences were found between high and low water within each entrainment condition.

To test whether any correlation existed between tidal elevation and total

TIDAL SYSTEM				
TIDAL STATE	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
LOW WATER	23.37 ± 7.25	9.09 ± 3.50	30.45 ± 6.90	10
LW + 4	23.14 ± 13.69	9.13 ± 7.93	34.10 ± 15.06	9
HIGH WATER	15.62 ± 9.91	8.08 ± 3.21	28.60 ± 11.74	7
LW + 8	16.55 ± 7.96	7.81 ± 4.03	22.98 ± 10.56	7
LOW WATER	20.73 ± 10.71	7.04 ± 2.49	27.34 ± 9.12	10

EXPOSED TIDAL SYSTEM				
TIDAL STATE	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
LOW WATER	29.54 ± 16.02	9.11 ± 6.84	35.10 ± 20.26	9
LW + 4	19.52 ± 12.35	6.47 ± 5.11	25.60 ± 15.03	10
HIGH WATER	17.90 ± 12.08	4.98 ± 3.17	24.78 ± 13.90	9
LW + 8	20.25 ± 6.74	7.37 ± 2.81	28.91 ± 7.88	7
LOW WATER	29.88 ± 6.69	8.88 ± 2.97	39.24 ± 7.58	10

ATIDAL/CONTROL SYSTEM				
TIDAL STATE	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
LOW WATER	22.87 ± 6.80	6.87 ± 2.46	25.45 ± 11.00	7
LW + 4	22.67 ± 16.14	5.69 ± 3.42	27.01 ± 15.84	8
HIGH WATER	32.07 ± 11.66	10.25 ± 4.42	38.09 ± 17.98	7
LW + 8	24.491 ± 2.56	7.29 ± 4.34	29.19 ± 15.22	8
LOW WATER	31.36 ± 24.75	8.93 ± 5.80	39.63 ± 23.25	8

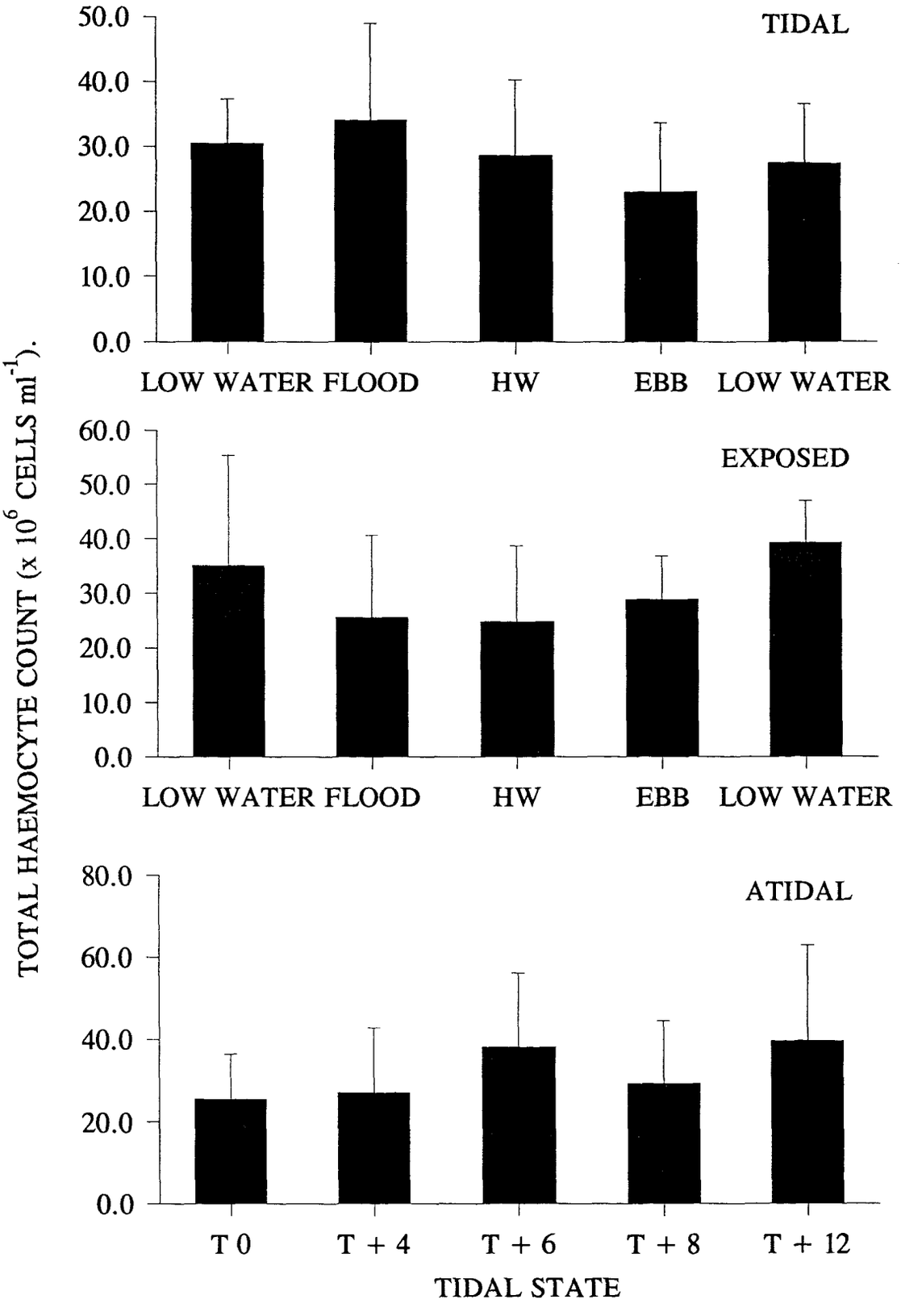

FIGURE 2.10 Fluctuations in total haemocyte count for crabs entrained to the three experimental protocols. Figure shows the mean ± one standard deviation of N observations as summarised in table 2.03.

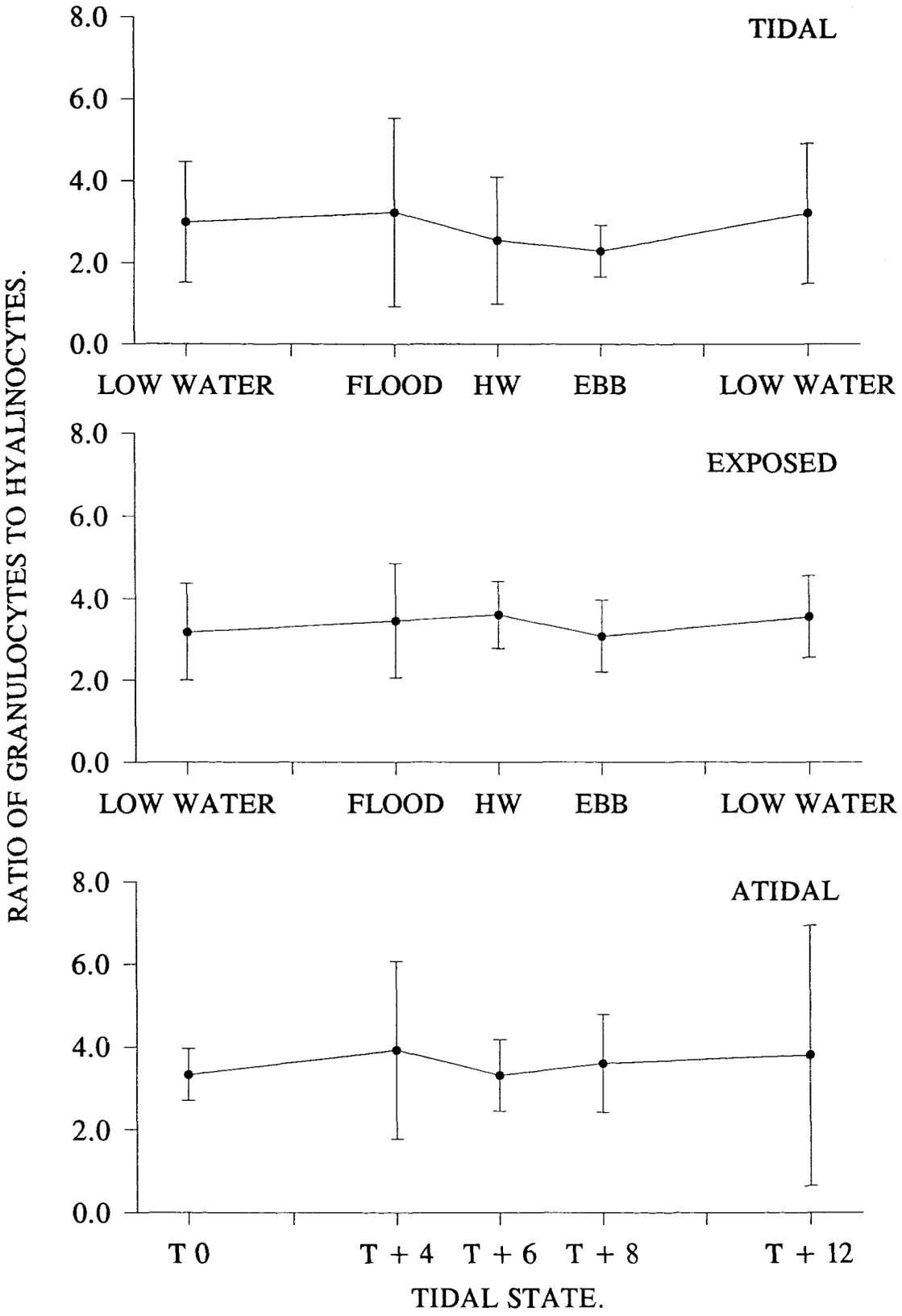

FIGURE 2.11 Changes in the ratio of granulocytes (semigranular and granular cells) to hyalinocytes with respect to tidal state and entrainment condition calculated using the data presented in table 2.03.

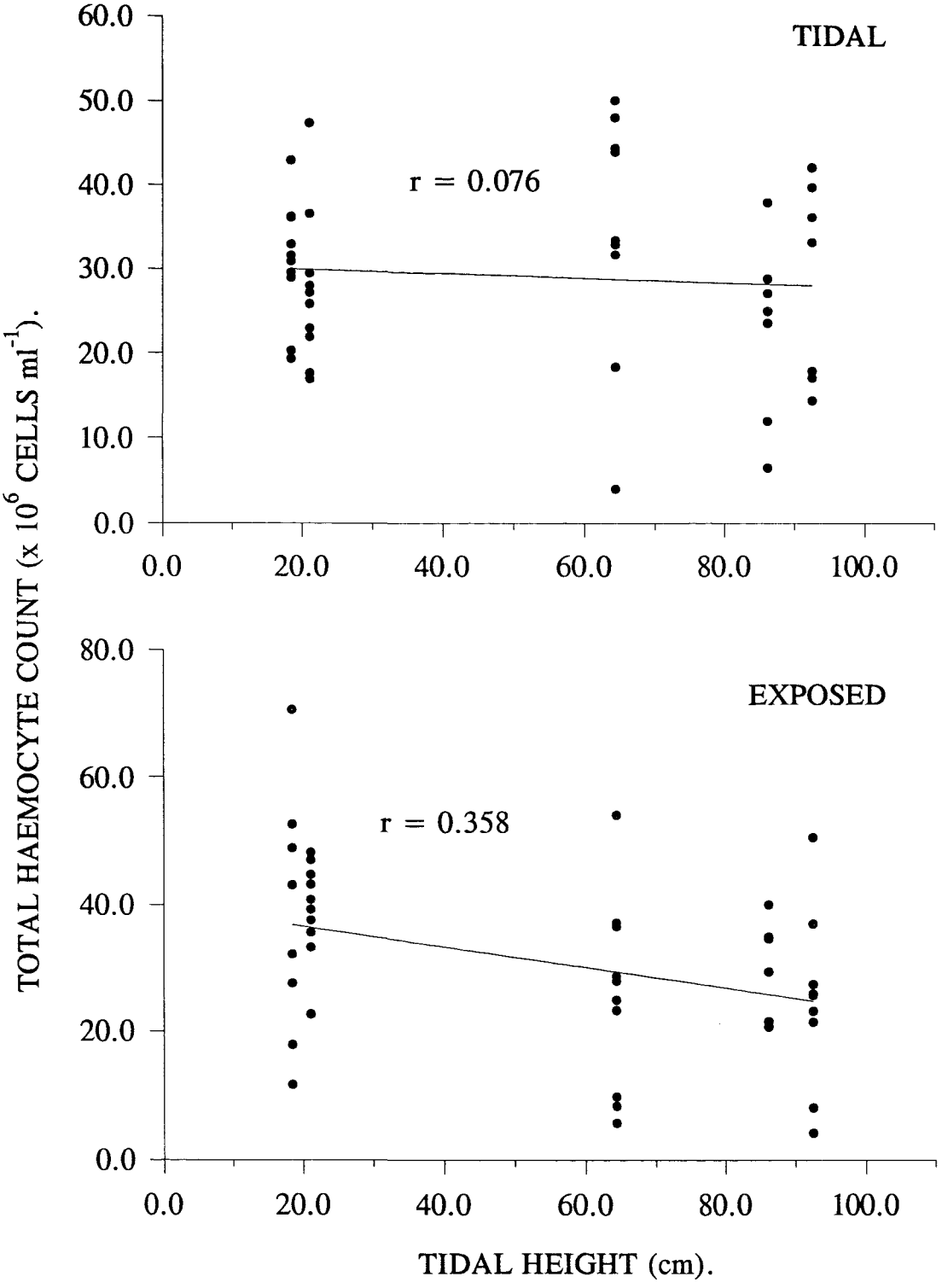

FIGURE 2.12 Relationship between total haemocyte count and tidal elevation for the tidal and exposed crabs. Regression relation indicated is calculated for the entire data set rather than the means shown in figure 2.10.

haemocyte population a first order linear regression was performed as shown in figure 2.12. There was a significant negative correlation between tidal elevation and THC in the case of the exposed system only (correlation coefficient, r = 0.358; $P < 0.05$). For the tidally entrained animals the cell population appeared to be independent of tidal elevation.

2.3.1.4 PHENOLOXIDASE ENZYME ACTIVITY.

The activity of the humoral defence enzyme phenoloxidase has been summarised for each exposure condition in table 2.04 and in figures 2.13 and 2.15. From table 2.04 it can be seen that phenoloxidase enzyme activity varied from 155.3 $\pm$ 120.8 to 424.6 $\pm$ 480.3 units per mg. protein in the activated samples and from 2.5 $\pm$ 2.3 to 23.3 $\pm$ 22.1 in the controls.

Considering the activated samples first it is evident that with the tidally entrained organisms there was an decrease in enzyme activity per mg. protein with increasing hydrostatic pressure whilst the opposite was evident in the tidally exposed individuals, figure 2.13.

No significant differences in phenoloxidase activity were found across the three entrainment conditions using one way ANOVA or within a single exposure system using *t* tests and Mann-Whitney Rank Sum tests for parametric and non parametric data respectively. The opposite trends shown in the tidal and exposed systems are highlighted in figure 2.14 which shows the linear regression relationships between phenoloxidase activity and tidal elevation. There was a significant negative correlation between the two variables for the tidally entrained crabs (correlation coefficient, r = 0.338; $P < 0.05$) whilst the relationship was positive in the case of the tidally exposed crabs.

Phenoloxidase enzyme activity in the control samples was very much less than for the trypsin activated HLS and never represented more than 20% of the activity of the samples treated with trypsin elicitor, figure 2.15. In both the tidally entrained and tidally exposed organisms there was no significant correlation

TIDAL STATE	EXPOSURE CONDITION								
	TIDAL			EXPOSED TIDAL			ATIDAL/CONTROL		
	SAMPLE	CONTROL	N	SAMPLE	CONTROL	N	SAMPLE	CONTROL	N
LOW WATER	424.6 ± 480.3	7.7 ± 7.7	7	158.3 ± 120.3	2.5 ± 2.3	6	406.3 ± 467.6	14.6 ± 27.0	6
LW + 4	337.4 ± 327.2	8.7 ± 8.5	7	283.0 ± 168.1	8.6 ± 5.9	7	410.9 ± 355.7	9.4 ± 9.3	7
HIGH WATER	212.9 ± 142.5	9.5 ± 5.6	7	348.3 ± 430.6	20.7 ± 24.8	7	173.4 ± 98.1	3.0 ± 2.5	7
LW + 8	155.3 ± 120.8	8.3 ± 10.6	6	237.1 ± 214.7	6.1 ± 6.3	7	250.1 ± 288.1	7.0 ± 9.2	9
LOW WATER	371.0 ± 329.6	23.3 ± 22.1	9	229.9 ± 221.6	12.1 ± 7.3	8	210.3 ± 142.2	17.3 ± 17.7	9

TABLE 2.04 Variation in phenoloxidase enzyme activity (units. mg protein^{-1}) with exposure condition and tidal state. All values represent the mean ± one standard deviation of N observations (one observation representing a pooled sample from two crabs, two entrainment runs performed for each exposure condition), and the atidal series represents the control environment.

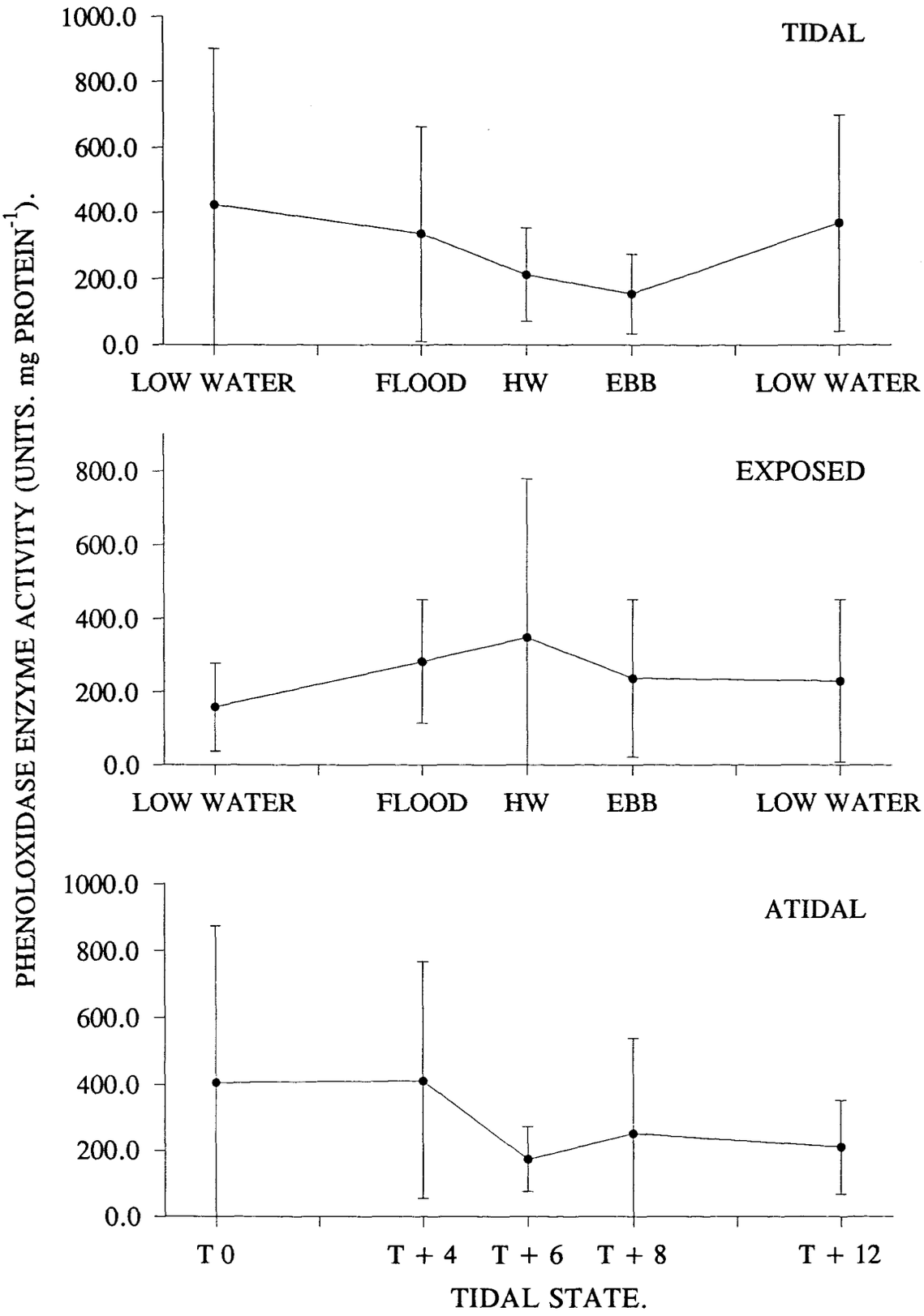

FIGURE 2.13 Variation in phenoloxidase enzyme activity (activated HLS) with respect to tidal state and entrainment condition. Data presented as summarised in table 2.04.

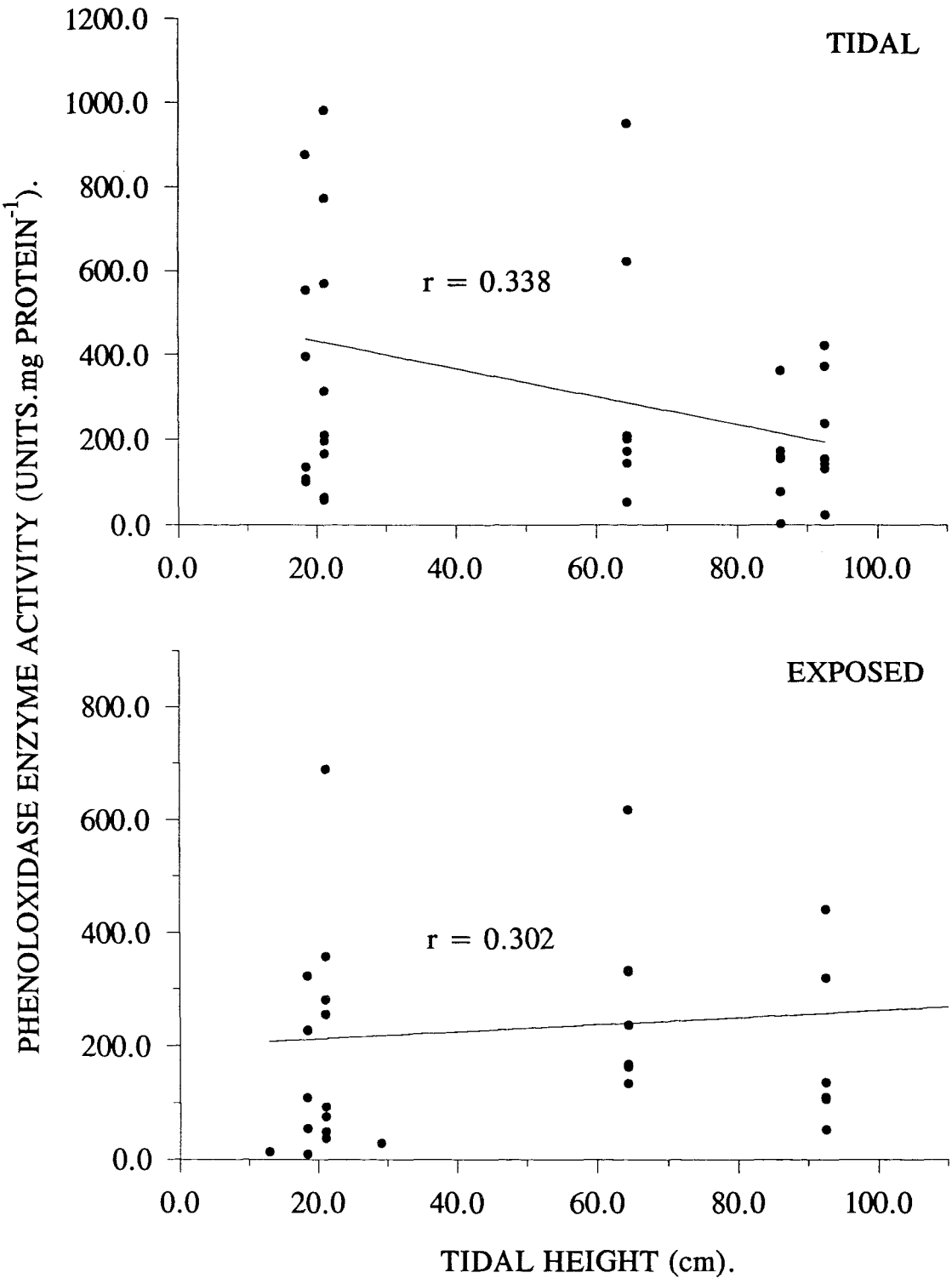

FIGURE 2.14 Relationship between phenoloxidase enzyme activity (activated HLS) and tidal height for the tidal and exposed crabs. The calculated regression relation is for the entire data set rather than the means presented in figure 2.13.

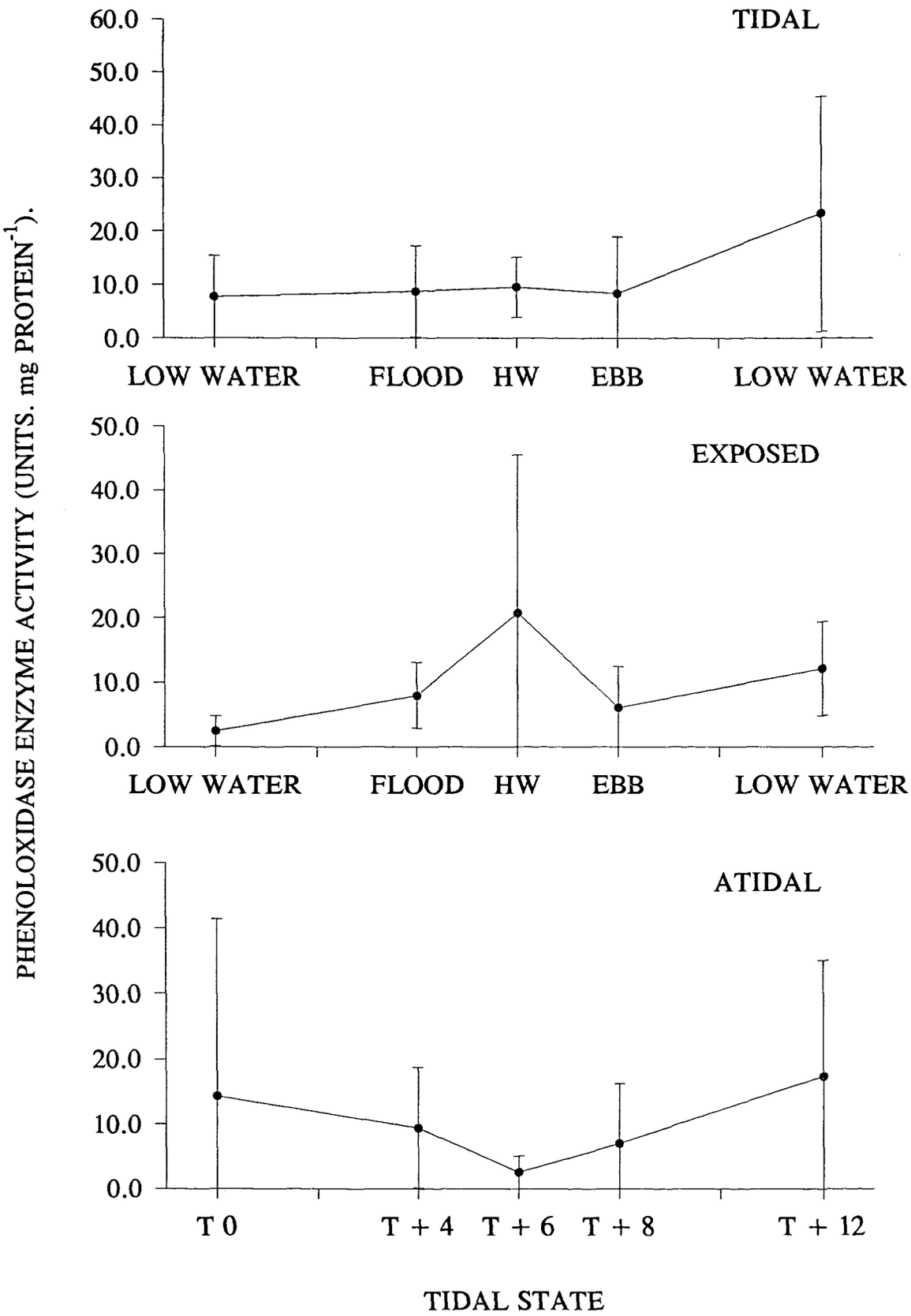

FIGURE 2.15 Phenoloxidase enzyme activity (control HLS) with respect to tidal state and entrainment condition. Data presented is as shown in table 2.04.

between tidal elevation and enzyme activity in the controls.

Kruskal-Wallis tests and subsequent Dunn's comparisons between the control entrainment and the two tidal entrainments revealed a significant ($P < 0.05$) elevation in enzyme activity at high water in both instances. Comparisons of changes in enzyme activity within each entrainment condition again proved inconclusive in all cases except the tidally exposed system (Mann-Whitney Rank Sum test, $P < 0.05$). In this environment there was a significant increase in enzyme activity at high water peaking at 20.7 $\pm$ 24.8 units per mg. protein.

2.3.2 MEASUREMENTS OF CARDIAC ACTIVITY.

Table 2.05 and figure 2.16 show the mean ($\pm$ one standard deviation, N = 6 crabs) heart rate at different tidal states for each entrainment condition, indicating that the mean heart rate varied about 100 beats per minute in all three entrainment conditions. No significant differences in the mean heart rate were detected across the three entrainment conditions using one way ANOVA or Kruskal-Wallis tests. Similarly no significant difference in mean heart rate between low and high water was found for any entrainment condition (*t* tests and Mann-Whitney Rank Sum Tests).

As previously indicated in section 2.2.3 the output voltage from the impedance coupler can be used to indicate the size of the cardiac contraction and table 2.05 and figure 2.17 show the mean rms output voltage (N = 6 crabs) from the impedance pneumograph. Again as with the heart rate data no significant differences in voltage output were found between the three exposure conditions or within a single system. This would indicate that the size of the cardiac contraction was independent of aerial exposure and tidal forcing in this experimental sequence. Both sets of data have large standard deviations which demonstrates extreme interindividual variability in cardiac activity, both in terms of the number of beats per minute and in the size of each heart contraction. In light of this variability and as the increase in heart rate associated with periods of high locomotor activity amounts to fluxes of only 15-20% (De Wachter, pers. comm.) the heart traces were also analysed individually. However, even when the data for each organism were analysed separately, without recourse to statistical methods, no consistent trends were evident, the heart rate appearing to be independent of tidal elevation.

TIDAL STATE	EXPOSURE CONDITION					
	TIDAL		EXPOSED TIDAL		ATIDAL/CONTROL	
	HEART RATE (BPM)	OUTPUT VOLTAGE (VOLTS)	HEART RATE (BPM)	OUTPUT VOLTAGE (VOLTS)	HEART RATE (BPM)	OUTPUT VOLTAGE (VOLTS)
LOW WATER	81.0 ± 34.7	0.19 ± 0.07	109.1 ± 42.3	0.26 ± 0.10	90.3 ± 39.7	0.19 ± 0.08
LW + 1	88.0 ± 48.1	0.25 ± 0.11	105.2 ± 49.3	0.29 ± 0.06	74.0 ± 32.7	0.24 ± 0.17
LW + 2	103.3 ± 37.9	0.23 ± 0.11	68.9 ± 51.6	0.21 ± 0.12	90.0 ± 44.1	0.22 ± 0.17
LW + 3	75.7 ± 44.6	0.18 ± 0.13	94.7 ± 57.7	0.23 ± 0.13	78.6 ± 33.3	0.24 ± 0.20
LW + 4	83.3 ± 43.7	0.21 ± 0.10	115.1 ± 21.0	0.21 ± 0.05	70.1 ± 30.5	0.17 ± 0.12
LW + 5	100.0 ± 67.4	0.18 ± 0.13	82.1 ± 36.9	0.15 ± 0.04	72.7 ± 30.5	0.19 ± 0.12
HIGH WATER	129.3 ± 58.4	0.24 ± 0.14	104.5 ± 39.3	0.17 ± 0.04	96.0 ± 63.1	0.16 ± 0.13
LW + 7	111.2 ± 81.4	0.18 ± 0.10	65.3 ± 21.2	0.13 ± 0.038	66.1 ± 24.4	0.16 ± 0.13
LW + 8	85.0 ± 39.4	0.16 ± 0.11	90.2 ± 21.0	0.15 ± 0.06	77.3 ± 43.9	0.16 ± 0.12
LW + 9	116.3 ± 72.8	0.16 ± 0.11	72.6 ± 48.6	0.18 ± 0.08	73.5 ± 44.1	0.17 ± 0.10
LW + 10	99.7 ± 45.0	0.15 ± 0.11	121.6 ± 29.1	0.22 ± 0.04	88.9 ± 56.0	0.16 ± 0.14
LW + 11	111.2 ± 81.0	0.17 ± 0.11	99.6 ± 34.0	0.19 ± 0.07	55.8 ± 18.1	0.16 ± 0.12
LOW WATER	102.2 ± 62.6	0.19 ± 0.18	90.0 ± 26.7	0.17 ± 0.07	75.1 ± 22.3	0.16 ± 0.15

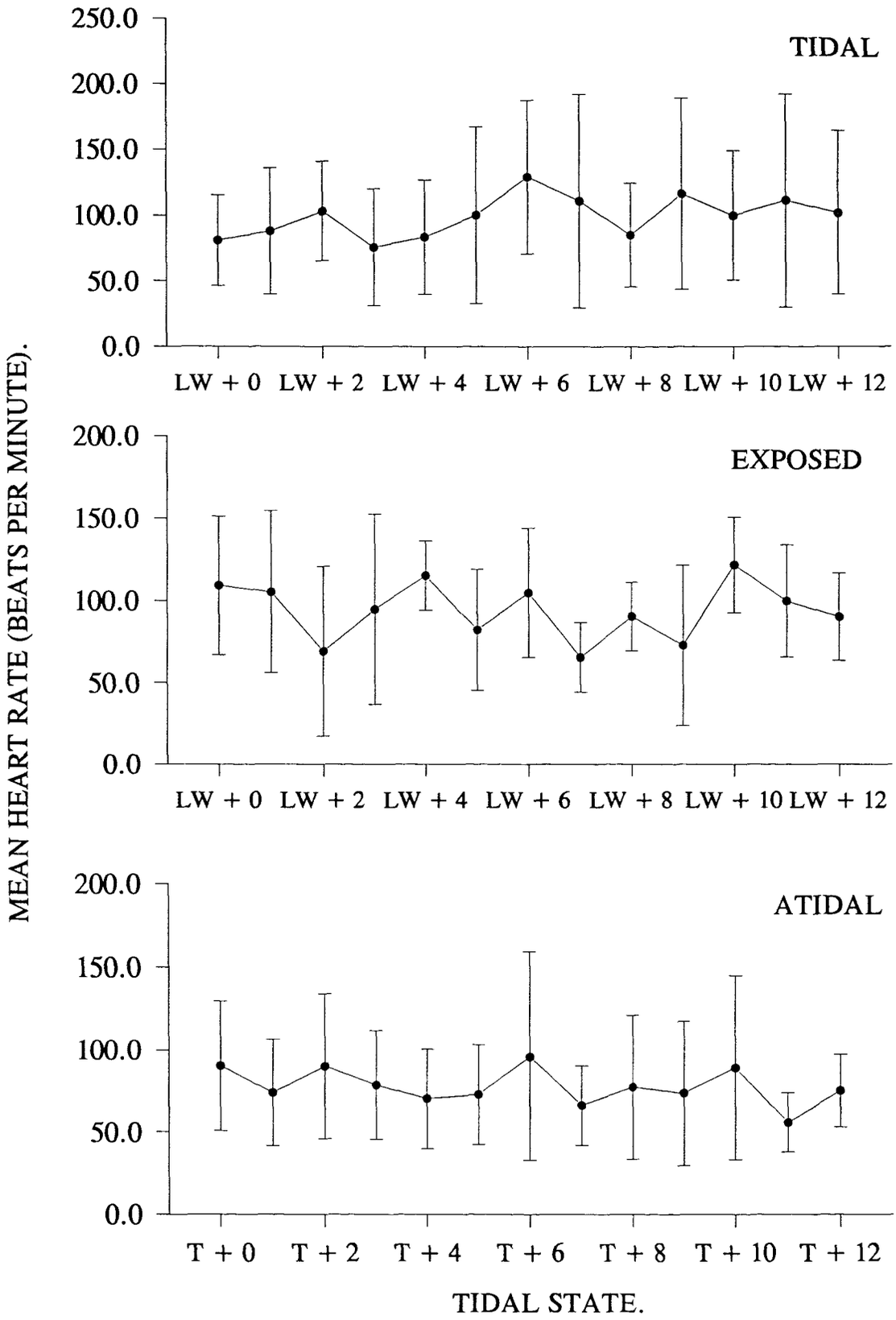

FIGURE 2.16 Changes in the cardiac activity in crabs relative to their entrainment condition and the tidal state expressed as beats per minute. Figure shows the mean ± one standard deviation of six observations in each case.

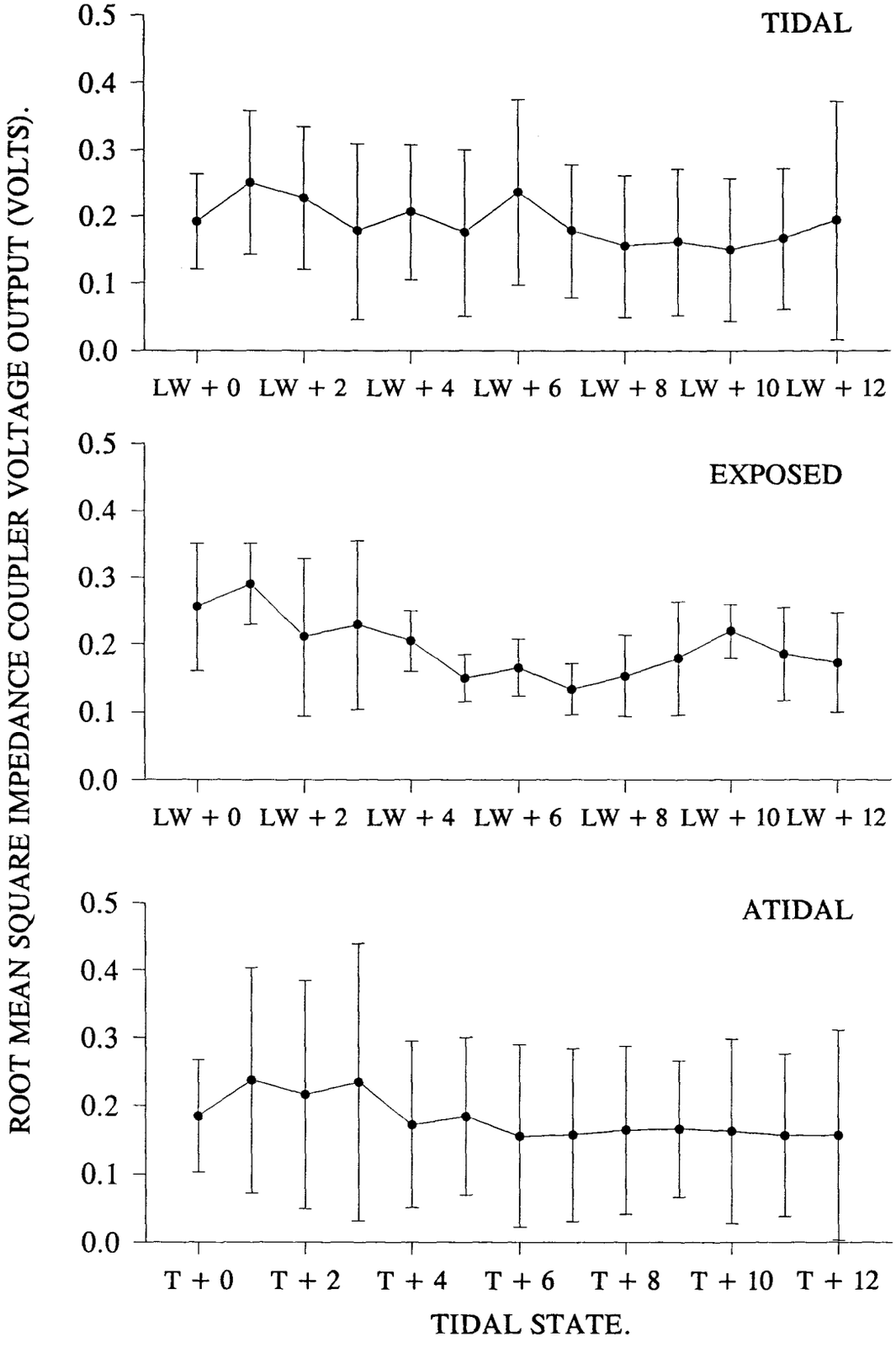

FIGURE 2.17 Variation in the impedance coupler output voltage, indicative of the amplitude of the cardiac contraction, as discussed in the text. Figure shows the mean ± one SD. of six observations for each entrainment condition.

2.4 DISCUSSION.

This chapter has summarised an investigation into tidal baseline variability in the measured indices of organism condition in *Carcinus maenas* which has provided important and novel data necessary for the implementation of these indices as markers of artificially induced stress.

2.4.1 METABOLIC INDICES OF ORGANISM CONDITION.

The data presented in this chapter indicate that there were predictable cycles of metabolic activity in *Carcinus maenas* associated with the tidal entrainment and also with repeated and regular aerial exposure. More specifically for the tidally entrained crabs it has been shown that there was elevated haemolymph sugar at low water and minimum concentrations at high water. Rajan *et al.* (1979) and Williams (1985) have also reported elevated haemolymph sugar in *C. maenas* during low water (23.3 $\pm$ 2.2 and 31.9 $\pm$ 5.7 mg. per one hundred ml. respectively) and decreased concentrations at high water (13.4 $\pm$ 1.9 and 21.7 $\pm$ 4.8 mg. per one hundred ml. respectively). High water has been recorded (Naylor, 1988; Reid & Naylor, 1989) as a period of increased locomotor activity and it is possible to conclude from the available data that the reduction in free haemolymph sugar represents its consumption in oxidative metabolism and removal from circulation. This conclusion is supported by current data showing elevations of the metabolic end product hydrogen peroxide approaching high water. Indeed there is a significant negative correlation ($r = 0.597$) between haemolymph sugar and hydrogen peroxide concentrations for all individuals irrespective of entrainment condition.

Hyperglycaemic events have been recorded for a variety of marine invertebrates by a number of workers and have usually been correlated with stressful events such as electric shock (in the mollusc *Aplysia*, Ram & Young, 1992), handling stress (in the lobster *Homarus americanus*, Telford, 1968) and metal exposure (in *C. maenas*, Hansen *et al.*, 1992). As has been discussed in the introductory chapter these elevations are superimposed on natural variation in

haemolymph sugar concentration, occurring even in "unstressed" individuals. It is apparent from the data presented in this chapter that any hyperglycaemia associated with induced stress in sublittoral estuarine and coastal crustaceans will be superimposed on tidally associated variability.

For the tidally exposed crabs the trends in haemolymph sugar and hydrogen peroxide are reversed with low sugar and high hydrogen peroxide concentrations found at low water from which it is apparent that the crabs were more metabolically active during periods of aerial exposure. The data collected in this present study appear anomalous in that they conflict with earlier reports of hyperglycaemic responses to aerial exposure in crustaceans (Roche & Dumazert, 1935; Kleinholz & Little, 1949; Johnson & Uglow, 1985; and Santos & Keller, 1993). These workers reported elevated sugar concentrations after exposure to air combined with, in some cases, an increase in the levels of circulating lactate. Santos & Keller (1993) concluded from their observations that the organisms were suffering hypoxia and respiring anaerobically and the elevation in sugar concentration was explained in terms of a reduced cellular uptake of glucose from haemolymph circulation. The discrepancy between the present investigation and earlier publications could be associated with the fact that these earlier workers used animals subjected to a single period of aerial exposure whereas for the present study crabs were repeatedly exposed to air for two hours every twelve hours over incubations of up to two weeks. It can be hypothesised that this repeated exposure may have led to a physiological adaptation of the organisms that included a reduction of cardiac activity during periods of emersion. Crustaceans have an open circulation with haemolymph sinuses rather than vessels. If the heart were to slow sufficiently then haemolymph would collect in these sinuses, leading to a local depletion of glucose and a build up of hydrogen peroxide. On reimmersion, when the cardiac activity increased the peripheral circulation would improve, restoring the haemolymph sugar concentration and at the same time removing the accumulated hydrogen peroxide. To test this idea a subsequent investigation of cardiac activity was undertaken as discussed below.

The levels of haemolymph sugar measured in the present study represent a "snapshot" of the internal environment of the crab and show nothing of the fluxes

of sugars due to the inter-conversion of glucose and glycogen and the uptake and release of sugars from tissues. Rajan *et al.* (1979) and Williams (1985) have indeed suggested that the cycling evident in tidally entrained animals is not due to glucose but to the presence of other oligosaccharides. Rajan *et al.* (1979) reported no tidal cycling of glucose in tidally entrained organisms, with the changes in the anthrone - responsive sugars being affected by fluctuations in the remaining oligosaccharides: fucose, fructose, maltose and maltotriose. In contrast Santos & Keller (1993), working with *C. maenas* and the crayfish *Orconectes limosus,* recorded fluctuations in glucose alone. A further possibility, suggested by Williams (1985), is that the cyclic pattern observed in both the tidally entrained and exposed crabs merely represents an inter-conversion between oligosaccharides and polysaccharides. Johnstone & Spencer-Davies (1972) reported that only twenty percent of the total carbohydrate in *C. maenas* is represented by low molecular weight sugars, the remaining 80% being made up of polysaccharides. It would only take a small change in the polysaccharides to bring about quite large changes in the levels of the anthrone - responsive sugars.

With the current data relating to changes in the *total* haemolymph sugar concentrations it was impossible to determine which components of the circulating carbohydrate pool were responsible for the observed predictable cycles and in light of this it may have proved more useful to measure individual sugars using several different assays rather than a single measure. Unfortunately at the time of the entrainment study no suitable assays had been developed to measure any of the other component sugars.

Superoxide production and the concomitant generation of hydrogen peroxide have been reported on a number of occasions for marine invertebrates and is widely considered to constitute a non specific immune defence in molluscs (Nakamura *et al.*, 1985; Dikkeboom *et al.*, 1987; and Hawkins *et al.*, 1993). For crustaceans such as *C. maenas* however the data are less conclusive. White *et al.* (1985) concluded following their studies that superoxide production was unlikely to occur amongst the Crustacea, a view that conflicts with the recent *in vitro* study of Bell & Smith (1992) which suggested that *C. maenas* hyalinocytes may produce

superoxide ions in the presence of bacterial pathogens. Supplementary evidence to support the arguments of Bell & Smith (1992) has recently been published by Song & Hseih (1994) for the tiger shrimp *Penaeus monodon*. However, whilst it is feasible that a proportion of the hydrogen peroxide measured may be due to hyaline cell superoxide production following pathogenic stimulation the current data collected would suggest that in this laboratory study the majority of the hydrogen peroxide measured in *C. maenas* represents the end product of routine metabolism.

2.4.2 INDICES OF IMMUNOCOMPETENCE.

It is evident from the data collected in the present study that regular changes in hydrostatic pressure can entrain cycles of haemocyte population within the haemolymph of *Carcinus maenas*. In tidally entrained crabs there was a tendency for there to be more haemocytes circulating within the haemolymph around high water and lower densities around low water. For the exposed crabs the tidally associated trends were reversed in a similar manner to that described for the metabolic indices discussed above. Truscott & White (1990) have also reported cyclical changes in the total haemocyte population of *C. maenas* taken from the sublittoral region of the Menai Straits, N. Wales, with elevated haemocyte densities being recorded at high water. In their work haemocyte populations fluctuated around a mean of 20×10^7 cells per organism which is an order of magnitude greater than in this study. Truscott & White (1990) measured THC as: "cells per whole organism" as opposed to: "cells per ml." as measured in this study. Further to this in the same paper a positive correlation between cell number and tidal range was also demonstrated. In this laboratory - based study changes in water depth were limited to one metre, the crabs used by Truscott & White (1990) were taken directly from the field where they were subjected to a regular tidal excursion of four metres. It is therefore conceivable that the crabs used in the present study would have demonstrated lower cell densities compared to those used by Truscott & White (1990).

The difference in tidal excursion offered to these two populations may also

go some way to explain the weak rhythmicity pattern of THC recorded in the tidally entrained crabs in this study. Specimens taken from the environment by Truscott & White had elevated THC's at high water (approximately 28 x 10^7 cells per organism) and reduced populations at low water (18 x 10^7 cells per organism). In this study there was a slight rise in haemocyte population towards high water but the trend was inconsistent. If the change in water depth had been greater then the fluxes in THC may have been more consistent. Having said this, in the case of the tidally exposed organisms the combination of a metre change in water depth combined with regular periods of exposure to air was sufficient to produce a consistent negative correlation ($r = 0.358$, $P < 0.05$) between water depth and haemocyte population.

Truscott & White (1990) reported increases in haemocyte number following food intake and during periods of locomotor activity. As discussed earlier periods of locomotor activity have been reported to be entrained to high water in *C. maenas* and the observations of Truscott & White (1990) would appear to agree with the publications of Naylor and coworkers (Naylor 1988, and Reid & Naylor, 1989). The elevations in THC during periods of exposure to air seen in the present study would therefore appear anomalous as the organisms are inactive and so the cell populations would be expected to decrease. A possible explanation of these observations is that the physiological response of a reduced cardiac output during aerial exposure suggested earlier allowed the haemocytes to collect in the peripheral sinuses sampled in this study. This theory is supported by the differential haemocyte counts which suggest that the uptake and release of haemocytes to and from the circulation during a tidal cycle was not cell specific (i.e. there was no evidence of a preferential removal of either granulocytes or hyalinocytes) and not related to an immune response. This evidence supports the hypothesis that changes in cardiac activity, both in terms of the heart rate and stroke volume, may be associated with periods of emersion as discussed below.

Johnstone *et al.* (1971), Williams & Lutz (1975) and Bachau (1981) have suggested a role for haemocytes in carbohydrate metabolism. These workers provided evidence including the presence of glucose-6-phosphatase in haemocytes (Johnstone *et al.*, 1971) to suggest that crustacean haemocytes contain reserves of

glycogen formed from circulating glucose and so have questioned the role of the hepatopancreas as the site of carbohydrate regulation. In contrast Loret (1993) has recently cast doubt on this theory of "circulating hepatocytes" showing that even in the presence of the crustacean hyperglycaemic hormone (CHH) the cells failed to release their stored glycogen as glucose. In the current study there was no significant correlation (Pearson Product Moment Correlation Coefficient = -0.176, $P > 0.05$) between haemolymph sugar and circulating haemocyte population. It would seem from these conflicting views that further work is required to clarify the exact role played by crustacean haemocytes in carbohydrate metabolism.

Circatidal variability in the activity of the phenoloxidase enzyme has been demonstrated for the first time in this study (Hauton *et al.*, 1995). There was a significant negative correlation (r = 0.338) between enzyme activity and tidal height for the tidally entrained animals suggesting cyclical changes in immunocompetence. Truscott & White (1990) reported similar tidally associated rhythms in the total haemocyte count for freshly captured *C. maenas,* with peaks in haemocyte population occurring at high water and suggested that "increased haemocyte numbers at high water may provide an enhanced immune capability during periods of high activity, when the risk of wounding is greater." Söderhäll & Smith (1983) reported that the zymogen prophenoloxidase is activated only in *the plasma.* If more prophenoloxidase is released into the plasma during periods of high activity (high water) to enhance immunocompetence then the remaining cellular fraction, measured using this assay, would be reduced as demonstrated in the current study. Hawkins *et al.* (1993) have reported circatidal rhythmicity in a number of immune parameters in tidally entrained specimens of the American hard clam (*Mercenaria mercenaria*) including amoebocyte number and lysozyme activity, with peaks occurring at times of increased activity including feeding. Again it was concluded that this reflected an enhanced level of immunocompetence during a period of increased exposure to pathogens.

For the exposed animals the decreases in phenoloxidase enzyme activity at low water may represent physiological adaptations to repeated bouts of exposure to

air. It is conceivable that during aerial exposure the organisms may "switch off" energetically expensive metabolism including the phenoloxidase system. The formation of lactate through anaerobic metabolism during aerial exposure has previously been demonstrated (Santos & Keller, 1993) and it is conceivable that the energy generated from such pathways is insufficient to maintain the granulocyte store of prophenoloxidase. This however, remains an hypothesis that requires further validation.

By comparing figure 2.10 and figure 2.13 it can be seen that the trends evident for phenoloxidase enzyme activity do not correlate with changes in THC (Pearson Product Moment Correlation Coefficient = - 0.393, $P > 0.05$). As pointed out by Smith & Johnstone (1993) there is no reason to expect any consistent correlation between the two variables as the units of measurement of phenoloxidase activity do not directly refer to cell number. Phenoloxidase activity was measured in terms of a change in absorbance per milligramme of protein which does not directly correlate with haemocyte population.

Questions must also be raised regarding what "phenoloxidase enzyme activity" actually represents. According to the theory put forward by Söderhäll & Smith (1983) prophenoloxidase is released as a zymogen into the plasma where it is activated by a serine proteinase (see figure 1.01). During the preparation of the haemocyte lysate supernatant the plasma is discarded. As a consequence the measure of phenoloxidase activity only indicates the total amount of prophenoloxidase being stored in the granulocytes and does not indicate any of the activated phenoloxidase that was present in the plasma. Control activity is again derived from the cellular fraction and only shows any activated phenoloxidase that was present on the surface of the haemocytes superimposed upon any activation that took place during the assay procedure. Classically immunocompetence has always been assessed in terms of activated phenoloxidase activity derived from the cellular component and it would perhaps be more appropriate in future to measure phenoloxidase activity in the plasma to indicate the *in vivo* active fraction (Hauton *et al.*, 1995). A potential problem with the measurement of plasma phenoloxidase is the melanisation of the samples during the initial centrifugation and any assay procedure would need to account for this oxidation (discussed further in Hauton *et*

al., 1995).

The procedure used to purify a sample of HLS was adapted from a number of methods and found necessary because of the experimental protocol adopted. The use of *Carcinus* anticoagulant was not initially advocated (Smith & Söderhäll, 1983) and seems to have been a later modification (Söderhäll & Smith, 1983), as does the sterilisation of the glassware to remove any bacteria and pyrogens (Jackson *et al.*, 1993; a modification independently adopted in this laboratory). Further to this the quick freezing of HLS in this study was a necessary modification due to the constraints of the sampling procedure which prevented the analysis of HLS until a day after collection. Pye (1978) has successfully performed phenoloxidase analyses using reconstituted freeze dried haemolymph samples - the only refinement required in this present study was to freeze the haemocytes in the final homogenizing buffer ensuring that any lysis and subsequent enzyme release during freezing would not lead to a loss of the enzyme in discarded supernatant.

2.4.3 MEASUREMENT OF CARDIAC ACTIVITY.

It has been suggested in the present study that the apparently anomalous haemolymph sugar, hydrogen peroxide and haemocyte population data recorded for the tidally exposed crabs may be explained in terms of a reduction in cardiac activity during periods of aerial exposure. It is hypothesised that during periods of hypoxia such as when aerially exposed, the heart may reduce output to conserve energy. If the heart did slow this would result in the haemolymph collecting in peripheral sinuses leading to local accumulations of haemocytes and hydrogen peroxide as well as local depletions of haemolymph sugars. Such reductions in heart rate with declining oxygen tension have been shown for the intertidal prawn *Palaemon elegans* (Morris & Taylor, 1985; Taylor & Spicer, 1989); the crabs *Cancer productus* (de Fur, 1988); and *Carcinus maenas* (Johnson & Uglow, 1985); and for the lobster *Homarus gammarus* (Hagerman, 1982). All of these authors used slightly different methods including single exposures and different exposure durations and consequently it was felt necessary to determine if a reduction in cardiac output was actually occurring in the present entrainments.

As discussed in section 2.3.2 the cardiac activity data, when analysed individually or according to entrainment condition, are inconclusive in demonstrating reductions associated with aerial exposure. In the earlier work on this subject all of the organisms were subjected to longer periods of aerial exposure - the shortest duration being four hours and it is conceivable that a repeated emersion of two hours every twelve hours is insufficient to induce a reduction in cardiac activity.

The work of Airriess & McMahon (1994) on *Cancer pagurus* has shown that cardiac action is a complex function which is not only determined by the heart rate but also by stroke volume. It was also demonstrated that there was a differential distribution of haemolymph around the body and in particular during hypoxic exposure there was a decrease in haemolymph flow via the anterior arteries to the antero - dorsal region concurrent with an increase in flow to the posterior and antero - ventral regions via the posterior aorta and the sternal artery. It is possible that redirected haemolymph flow during hypoxia may preferentially supply the peripheral sinuses sampled in this study and so explain the observations made relating to circulating haemocyte population. This idea does however, remain unverified and would require validation.

The use of Fourier analysis, whilst beneficial in this instance because of the large volume and format of the data, is not without its problems. One feature of the crustacean myogenic heart is its apparent ability to undergo periods of cardiac arrest (Burnett & Bridges, 1981; Morris & Taylor, 1985; and de Fur, 1988) usually associated with environmental hyperoxia. Cardiac arrests were never recorded during this present study but had such traces been analysed using Fourier analysis, two dominant wave forms would have been isolated, one of a very high frequency and one a low frequency, neither of which would accurately reflect the true heart rate.

It is clear from the data presented in this chapter that there are predictable, entrainable circatidal rhythms in a number of metabolic and immunological parameters for the shore crab *Carcinus maenas*. This inherent variation must be accounted for when analysing stress indices in organisms collected from regions in

which tidal forcing occurs. As discussed in the introduction tidal forcing is only one of a number of impinging environmental variables; another important influence is that of the seasonal cycle and this forms the basis of the next chapter.

CHAPTER THREE

SEASONAL VARIATION IN THE INDICES OF METABOLIC STATUS AND IMMUNOCOMPETENCE IN *Carcinus maenas*

CHAPTER THREE

SEASONAL VARIATION IN THE INDICES OF METABOLIC STATUS AND IMMUNOCOMPETENCE IN Carcinus maenas

3.1 INTRODUCTION.

The studies described in chapter two indicated that one of the most important facets of the estuarine environment is the regular change in hydrostatic pressure associated with the tidal cycle. The immunological and metabolic consequences of such hydrostatic forcing on *Carcinus maenas* were demonstrated and the implications of this baseline variability in terms of biomonitoring were discussed. Clearly, another important feature of shallow water environments is the influence of the regular cycle of seasons and the changes in *in situ* environmental conditions which result. It is evident for *C. maenas*, and indeed any potential marine biomarker, that the nature of variation in organism condition associated with the seasonal cycle must be elucidated before any constructive analysis of an animal's response to "artificially" imposed stress can be carried out. To this end the present chapter reviews an investigation into seasonally related variability in the measured indices of metabolic status and immunocompetence in the shore crab.

C. maenas, as a poikilothermic invertebrate will, by nature, be expected to operate at a reduced biochemical rate in the cold. This however, is an oversimplification of the changes that one might find as seasonal fluctuations in environmental conditions influence a number of other parameters as well as temperature. Further to this *C. maenas*, an estuarine species, is essentially eurytopic (Bayne *et al.*, 1985) and as a consequence demonstrates an ability to compensate for wide variations in environmental condition as discussed by Mantel (1983).

Historically seasonal influences on the condition of marine invertebrates

have been investigated by a number of workers. More recently Brooks (1994) has reported a decrease in the immunocompetence of a Solent population of the European flat oyster (*Ostrea edulis*) during the summer months when temperatures rose above 10°C, as shown by a reduced ability to produce superoxide anions and a fall in haemolymph lysozyme activity. At the same time *in vitro* studies carried out on individuals collected directly from the Solent demonstrated a reduction in the phagocytosis of the Gram negative bacteria *Listonella (= Vibrio) anguillarum* during the summer. This work in essence, contradicted the earlier work of Muramoto *et al.* (1991), who had recorded increased agglutinin activity in the acorn barnacle (*Megabalanus rosa*) during the summer months compared with the winter, although whether this was a direct consequence of temperature or was due to other physiological factors was not determined by the authors.

In decapod crustaceans Chisholm (1993) has reported that during the summer months there were reductions in the haemocyte counts of a population of *C. maenas* collected in Scotland and also that the non-specific antibacterial activity of *C. maenas* was reduced at extremes of temperature. The earlier laboratory work of Truscott & White (1990) had demonstrated an increase in the number of circulating haemocytes following exposure to a gradual increase in temperature from 10°C to 20°C over a twelve hour period (rising from 32 x 10^7 haemocytes per animal to 51 x 10^7 haemocytes per animal) although whether this work can be reliably extrapolated to the natural environment remains debateable. The work by Truscott & White (1990) which suggests heightened immunocompetence during warm periods is in agreement with the earlier studies of Ravindranath (1977) on the crab *Emeritia asiatica*, and Dean & Vernberg (1966) on the fiddler crab (*Uca pugilator*) who also demonstrated that cold-acclimated animals had slower clotting times and lower plasma protein concentrations than those maintained at higher temperatures.

In contrast, the affects of seasonal change on the metabolic status of *C. maenas* have received only limited attention, with workers tending to focus on changes in laboratory maintained populations. One *in situ* study of the changes in biochemical composition of *C. maenas* associated with the seasonal cycle found

there to be no clearly defined changes in the carbohydrate composition of the hepatopancreas (Heath & Barnes, 1970), whilst the later work of Thabrew *et al.* (1971) showed that the glucose concentration within the gill tissue was highest between the months November to March and lowest during the summer - although these workers did emphasise the poor reproducibility of data between different animals. For the crabs *Uca pugilator* and *U. minax* Dean & Vernberg (1965) have reported an increase in haemolymph glucose following temperature increases both in the laboratory and field (ca. 10 milligramme percent at 10°C and 30 milligramme percent at 30°C). Similarly, Florkin (1960) reported an increase in oxygen consumption in the crabs *C. maenas* and *Eriphia spinifrons* and the amphipod *Gammarus locusta* on transferal from seawater to brackish water. Schwoch (1972) has discussed the synthesis of the glucose dimer trehalose in the crayfish (*Orconectes limosus*) and demonstrated a reduction in synthesis during the summer months (from values of 6.0 in the winter to 0.6mμ moles per minute per milligramme of protein in the summer).

It can be seen from the rather sparse and conflicting data that a comprehensive study of the response of *C. maenas* to seasonal environmental change is required. To this end crabs were sampled from the River Itchen every spring tide (negating spring/neap associated cycles as demonstrated by Dare & Edwards, 1981; and Naylor, 1985; and reviewed by Naylor, 1988) to determine natural variation in the measured parameters. At the same time the resolution of the sugar data was improved by measuring the concentration of the haemolymph sugars glucose and trehalose with two new assays. Ancillary measures of the changing environmental conditions were also recorded in an attempt to identify any potential correlations.

3.2 METHODS.

3.2.1 GENERAL EXPERIMENTAL PROCEDURE.

Haemolymph samples were collected from crabs in the field at high water every spring tide over a fifteen month period from 28th March 1994 to 28th June 1995. As a consequence of the limited volume of haemolymph available the metabolic indices (haemolymph sugars and hydrogen peroxide concentration) were measured every other spring tide alternating with the immunological indices (phenoloxidase enzyme activity and haemocyte counts).

Crabs were collected from the sublittoral using baited pots deployed in the River Itchen (50° 45.75' N, 1° 22.70'W; see figure 1.02). The sex and carapace width, in millimetres, of all sampled crabs were recorded after the haemolymph samples were collected and the crabs were returned directly to the estuary after bleeding. Haemolymph samples were taken from intermoult crabs via the unsclerotized membrane between the basiopodite and coxopodite of the cheliped using sterile syringes and 25 gauge needles.

At the same time as the haemolymph samples were taken ancillary environmental measurements were recorded. Water temperature and salinity were recorded on site and a water sample was collected for determination of the bacterial population and also the total suspended particulate and organic load.

3.2.2 BIOCHEMICAL AND IMMUNOLOGICAL METHODS.

For the seasonal study all of the metabolic measurements (haemolymph total sugar, glucose, trehalose and hydrogen peroxide) were determined from a single haemolymph sample in each case. Before deproteinising the haemolymph with a solution of 1M perchloric acid for the total sugar and trehalose determinations, two 0.1ml. aliquots of haemolymph were removed for the glucose and hydrogen peroxide assays. Due to the constraints of the assay procedures however, the immunological measures of phenoloxidase enzyme activity and haemocyte population were made on haemolymph from different organisms.

3.2.2.1 HAEMOLYMPH SUGAR DETERMINATION (ANTHRONE METHOD).

The concentration of total reducing sugars in the haemolymph of the sampled crabs was determined using the anthrone method (Roe, 1955) described in detail in section 2.2.2.1. The initial deproteinising of the haemolymph with perchloric acid was carried out immediately after bleeding on site. The treated samples were subsequently returned to the laboratory for analysis.

3.2.2.2 HAEMOLYMPH GLUCOSE DETERMINATION.

Haemolymph glucose was measured by adapting the method of Kunst *et al.* (1984). 0.1ml. of haemolymph was immediately deproteinised with 0.9ml. of a solution of 2% perchloric acid. This was carried out on site after which the samples were stable at room temperature for a maximum of three days (Kunst *et al.*, 1984). All samples were returned to the laboratory and were analysed on the same day.

Deproteinised samples were centrifuged at 3000rpm., equivalent to 750*g*, for 15 minutes to remove the cell debris and leave a clear supernatant. 50μl. of this supernatant was added to a mixture of 0.93ml. adenosine triphosphate (ATP) buffer (see appendix A.4), 10μl. of a solution of 100mM ATP and 5μl. of glucose-6-phosphate dehydrogenase (G-6-PDH). The absorbance (A_1) of the samples was then measured at 340nm using a Cecil CE 292 Digital Ultraviolet Spectrophotometer before 10μl of hexokinase (HK) was added. After ten minutes incubation at room temperature the new absorbance (A_2) was recorded. Sample standards (50mg. $100ml^{-1}$) and blanks were analysed simultaneously and were treated in an identical manner to the haemolymph samples. The experimental protocol is summarised in figure 3.01.

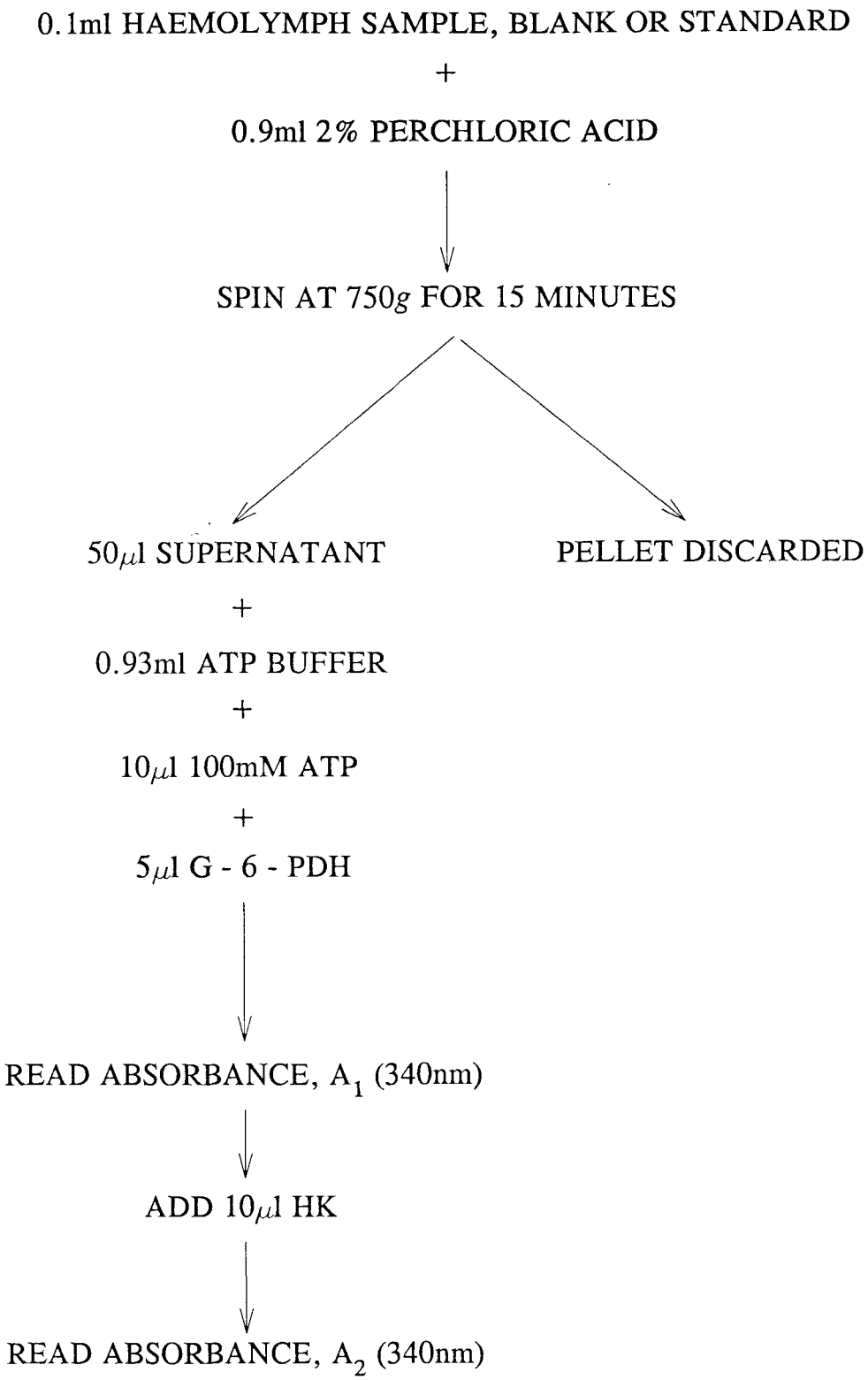

FIGURE 3.01 Flow diagram showing experimental protocol adopted for haemolymph glucose determination. Full explanation given in the text.

Haemolymph glucose concentration was calculated using the following relation:-

$$[GLUCOSE]\ (mg.\ 100ml^{-1}) = \frac{(A_2 - A_1)_{SAMPLE}}{(A_2 - A_1)_{STANDARD}} \times 50 \qquad (5)$$

3.2.2.3 HAEMOLYMPH TREHALOSE DETERMINATION.

Haemolymph trehalose was measured by adapting the method of Johnstone and Spencer-Davies (1972). A quantitative volume of the deproteinised haemolymph from the anthrone assay (see section 2.2.2.1) was digested in the presence of 0.1 volume of 1M sodium hydroxide at 100°C for one hour, using a Techne Dri-Block® DB-2A heating element. After incubation the solutions were made back up to one volume, using saturated benzoic acid solution. Whilst reducing sugars are labile to basic hydrolysis the α (1-1) linkage of trehalose remains stable under these conditions. Following digestion the trehalose was assayed using the anthrone method (Roe, 1955; see section 2.2.2.1)

This method was adopted after initial attempts involving an enzymic digestion failed to produce reliable data. Initially it was hoped that a commercial preparation of trehalase (EC 3.2.1.28, purified from porcine kidney by the Sigma Chemical Company Ltd.) could be used to breakdown the disaccharide trehalose to glucose which could subsequently be assayed as in section 3.2.2.2. A number of different assay procedures and buffers were tried based around Sigma's own trehalase calibration method (Jess, pers. comm.). The only assay which apparently produced some trehalose breakdown has been summarised in figure 3.02; even this assay however, generated unreliable and inconsistent data which always underestimated the concentration of the trehalose present in the standard.

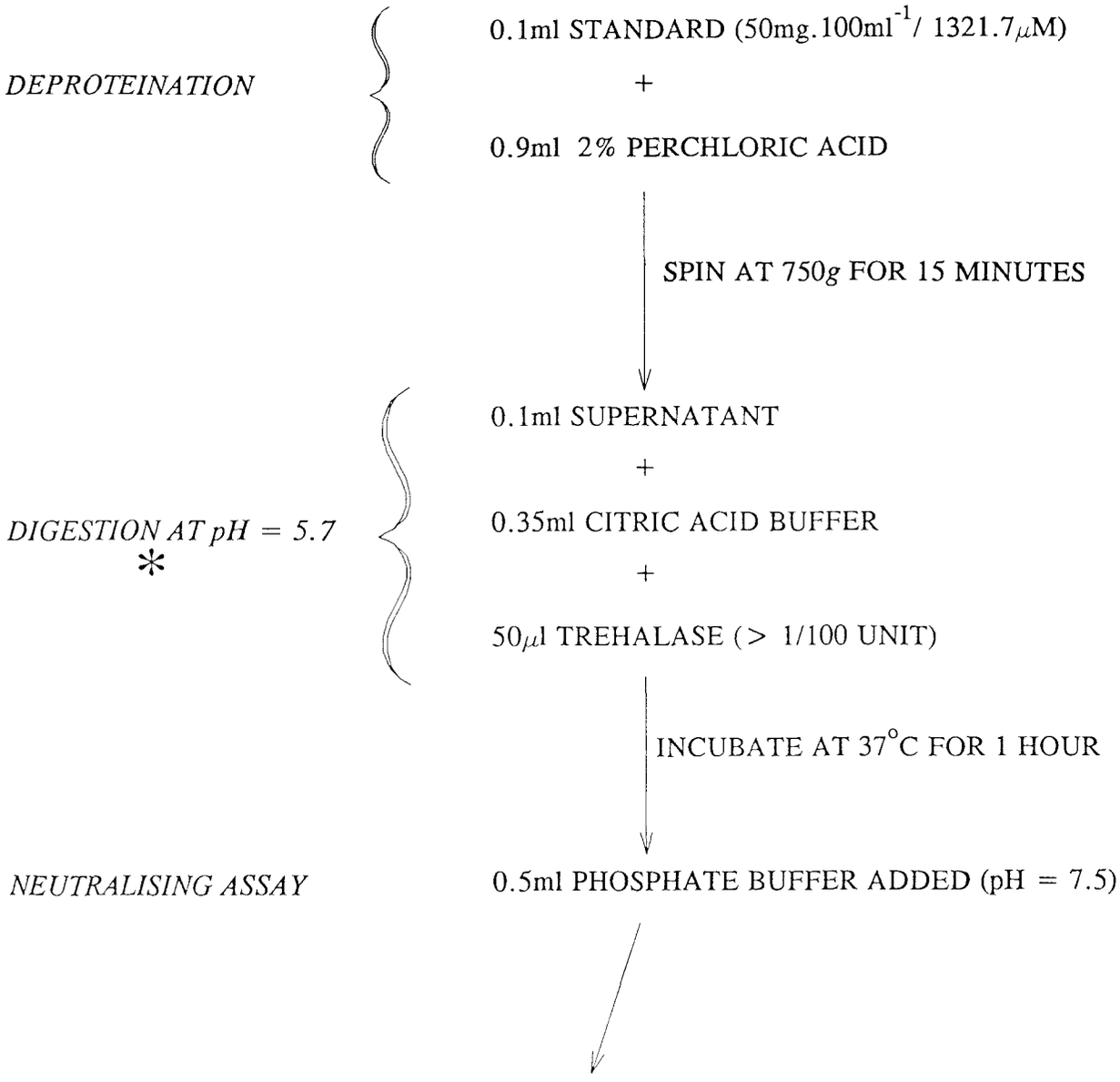

FIGURE 3.02 Flow diagram of the enzymic trehalose digestion assay.

* Digestion conditions: pH = 5.7, Temperature = 37°C, 50 fold dilution of the initial standard = 26.43μM in 0.5ml giving 0.0132μmoles of trehalose in assay. Enzyme activity: One unit converts 1μmole trehalose to 2μmoles of glucose per minute at 37°C. Therefore 1/100th of a unit would theoretically take ca. 1.5 minutes to breakdown the trehalose present.

3.2.2.4 HAEMOLYMPH HYDROGEN PEROXIDE DETERMINATION.

Hydrogen peroxide was measured by the method described in section 2.2.2.2. As before 0.1ml. of haemolymph was immediately deproteinised on site after collection using 0.1ml. of a solution of 1M perchloric acid.

3.2.2.5 TOTAL AND DIFFERENTIAL HAEMOCYTE COUNTS.

Counts of circulating haemocyte population were made as described in section 2.2.2.3.

3.2.2.6 PHENOLOXIDASE ENZYME ASSAY.

Phenoloxidase enzyme activity was determined as has been described previously (see section 2.2.2.4). For this study however, the volume of haemolymph withdrawn was increased to 1.0ml. and was diluted with 1.0ml. of citrate EDTA as an anticoagulant. The initial centrifugation and washing of the haemocyte pellet was performed on site before the samples were rapid frozen by immersion in liquid nitrogen. The frozen samples were then returned to the laboratory for subsequent analysis.

3.2.3 ANCILLARY ENVIRONMENTAL MEASURES.

Ancillary environmental data were measured from surface water samples only, as Antai (1990) has previously shown that there was no difference in the physical and chemical conditions between surface and bottom water in the estuary.

3.2.3.1 TEMPERATURE AND SALINITY.

The temperature and salinity were measured from surface water samples taken every two weeks at spring high water. Salinity was measured using a Reichert™ refractometer, and temperature with a mercury-in-glass thermometer.

3.2.3.2 SUSPENDED PARTICULATE AND ORGANIC LOAD.

Three replicate 500ml. samples of seawater were filtered through preweighed ashed Whatman™ 1.2μm GF/C filters. The filters were oven dried (70°C) to constant weight before being weighed again and ashed in a furnace for twenty-four hours at 550°C. The ashed filters were weighed a final time and the three different measures were used to calculate dry weight (total particulates) and ash free dry weight (organics).

3.2.3.3 BACTERIAL POPULATION STUDIES.

Routine measurements of seawater bacterial load were made at high water on every spring tide. Three 20μl. aliquots of the seawater sample were plated on general marine agar (GMA, see appendix C.1) and incubated for ten days at 18°C ($\pm$ 1°C). After the incubation period the number of bacterial colonies present on each plate were counted to give a measure of total viable count (TVC_{10}). Further to this a preliminary investigation of the bacterial species present was undertaken using two different approaches.

A selective agar media was employed to indicate the presence or absence of particular species. The *Listonella (= Vibrio)* selective medium (thiosulphate-citrate bile salt, TCBS) described by Kobayashi *et al.* (1963) was used to indicate the presence of any *Listonella* species (see appendix C.1). A *Listonella* positive reaction was indicated by a change in colour of the agar from brown to blue around each colony.

The second approach to bacterial identification used the Gram stain method (Stevens, 1982). Different bacterial species (identified by colony colour and

appearance) were grown up in general marine broth (see appendix C.1) and samples of the broth were taken to produce air dried bacterial smears. These smears were then stained according to the method of Stevens (1982) and examined under the bright field optics of a Zeiss™ KF2 microscope. Gram reaction and physical shape of the bacteria were used to identify the different species present.

Occasionally (< 5% of crabs sampled) the collected haemolymph had a milky white appearance, indicative of a gross bacterial infection (Johnson, 1983). Although this haemolymph was not used in any assay a sample was retained and plated out on GMA. Alsina *et al.* (1994) have more recently developed a *Listonella anguillarum* medium (LAM) which was also used to specifically indicate strains of this species - a positive reaction being indicated by a change in colour of the agar from blue to yellow around each colony due to fermentation of sorbitol (see appendix C.1). Further to this once the bacteria had grown out a sample was inoculated in general marine broth to produce air dried bacterial smears which were analysed using the Gram stain method described above.

3.2.4 STATISTICAL ANALYSIS OF DATA.

To avoid any problems associated with size linked differences in metabolic rate the carapace widths of all crabs sampled were measured. Significant differences in the mean size of crabs each spring tide were highlighted using one way ANOVA and subsequent Student Newman Keuls multiple comparisons (Kruskal-Wallis ANOVA and Dunn's multiple comparisons as a non parametric alternative). Whilst all of the data have been presented for completeness those months where the mean size of crabs sampled *was* significantly lower have been marked with an asterisk and the data collected has been removed from further analysis.

Having removed any possibly size related data the remaining measures were analysed using the ANOVA tests outlined above. Pearson Product Moment Correlations were performed to indicate any association between the measured indices and the environmental variables. This test was chosen in favour of first order linear regression as it was not always possible to assign a single independent and dependent variable.

3.3 RESULTS.

3.3.1 ENVIRONMENTAL PARAMETERS.

The change in water temperature and salinity during the period of this investigation have been presented in figures 3.03 and 3.04 respectively. Water temperature reached a maximum of 20.0°C on 18th July 1994 and subsequently declined during the autumn and winter to a minimum of 6.0°C on 30th January 1995. The general temperature fluctuations tended to be predictable with the cycle repeating itself during 1995. Salinity measures tended to be more variable as might be expected being a function of riverine inputs and precipitation. As can be seen from figure 3.04 however, salinity tended to be highest during the spring and autumn (April to late May 1994 and late September to late November 1994) with lower salinity occurring during the summer of 1994 and January to March 1995. The maximum and minimum salinity recorded during this study were 30.3‰ on 28th March 1994 and 10‰ on 15th February 1995 respectively.

Total particulate and organic loads in the water column have been indicated in figures 3.05 and 3.06 respectively. It is evident from these two figures that whilst the data were quite variable both particulate and organic loads in the water column tended towards a maximum (approximately 0.035 grammes per litre and 0.0125 grammes per litre respectively) during the late summer and autumn/winter of 1994, values for both being lower during January to June 1995. It can be seen by comparing figures 3.05 and 3.06 that there was a tendency for the two variables to correlate. Indeed as shown in table 3.01 there is a very significant ($P < 0.001$) positive correlation between total particulate and organic load in the water column. Further to this the organic contribution to the total particulate load remains constant throughout the study at approximately forty percent.

Figure 3.07 indicates the variation in bacterial population as determined from plating seawater samples onto GMA. It was evident that for most of the year the bacterial population ranged between 0 and approximately 7500 colonies per millilitre but during September and October 1994 very high and very variable densities were recorded (means of 9200 and 12050 colonies per millilitre

	TOTAL ORGANICS	TOTAL PARTICULATES	BACTERIA	SALINITY	TEMP.	PHENOLOXIDASE ACTIVITY	RATIO G:H	CELL COUNTS	H_2O_2	TREHALOSE	GLUCOSE
ANTHRONE SUGARS	0.027 *0.822* **72**	0.233 *0.049* **72**	-0.270 *0.022* **72**	-0.134 *0.261* **72**	0.157 *0.188* **72**	NOT TESTED	NOT TESTED	NOT TESTED	0.094 *0.394* **84**	0.046 *0.002* **41**	0.306 *0.027* **52**
GLUCOSE	0.301 *0.035* **49**	0.059 *0.689* **49**	-0.017 *0.908* **49**	0.045 *0.756* **49**	0.403 *0.004* **49**	NOT TESTED	NOT TESTED	NOT TESTED	-0.076 *0.785* **59**	-0.077 *0.701* **27**	
TREHALOSE	0.514 *0.005* **28**	0.544 *0.003* **28**	0.457 *0.014* **28**	0.468 *0.012* **28**	0.546 *0.003* **28**	NOT TESTED	NOT TESTED	NOT TESTED	0.349 *0.027* **40**		
H_2O_2	0.108 *0.315* **88**	0.036 *0.741* **88**	-0.174 *0.105* **88**	0.087 *0.420* **88**	0.105 *0.332* **88**	NOT TESTED	NOT TESTED	NOT TESTED			
CELL COUNTS	-0.360 *0.005* **59**	-0.077 *0.564* **59**	-0.053 *0.691* **59**	0.414 *0.001* **59**	-0.031 *0.817* **59**	NOT TESTED	0.303 *0.009* **73**				
RATIO G:H	-0.120 *0.363* **59**	0.030 *0.821* **59**	0.249 *0.057* **59**	0.112 *0.398* **59**	0.219 *0.096* **59**	NOT TESTED					
PHENOLOXIDASE ACTIVITY	-0.084 *0.519* **61**	0.051 *0.697* **61**	0.259 *0.044* **61**	-0.011 *0.931* **61**	0.141 *0.277* **61**						
TEMPERATURE	NOT TESTED	NOT TESTED	0.326 *0.004* 77	NOT TESTED							
SALINITY	NOT TESTED	NOT TESTED	0.309 *0.006* 77								
BACTERIA	0.269 *0.018* **77**	0.435 *<0.001* **77**									
TOTAL PARTICULATES	0.758 *<0.001* **26**										

TABLE 3.01 Correlations between the measured indices of organism condition and the environmental variables. Cell contents represent the correlation coefficient (normal text), *P* value (italics), and number of samples (bold). Significant comparisons have been shaded.

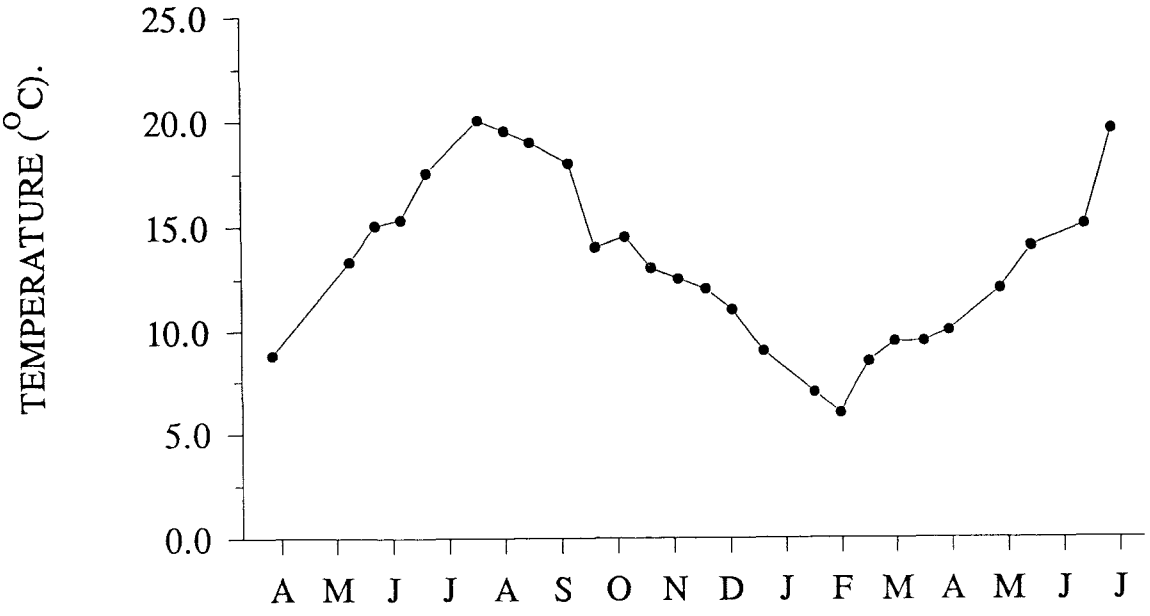

FIGURE 3.03 Seasonal variation in surface water temperature for the River Itchen during 1994/95.

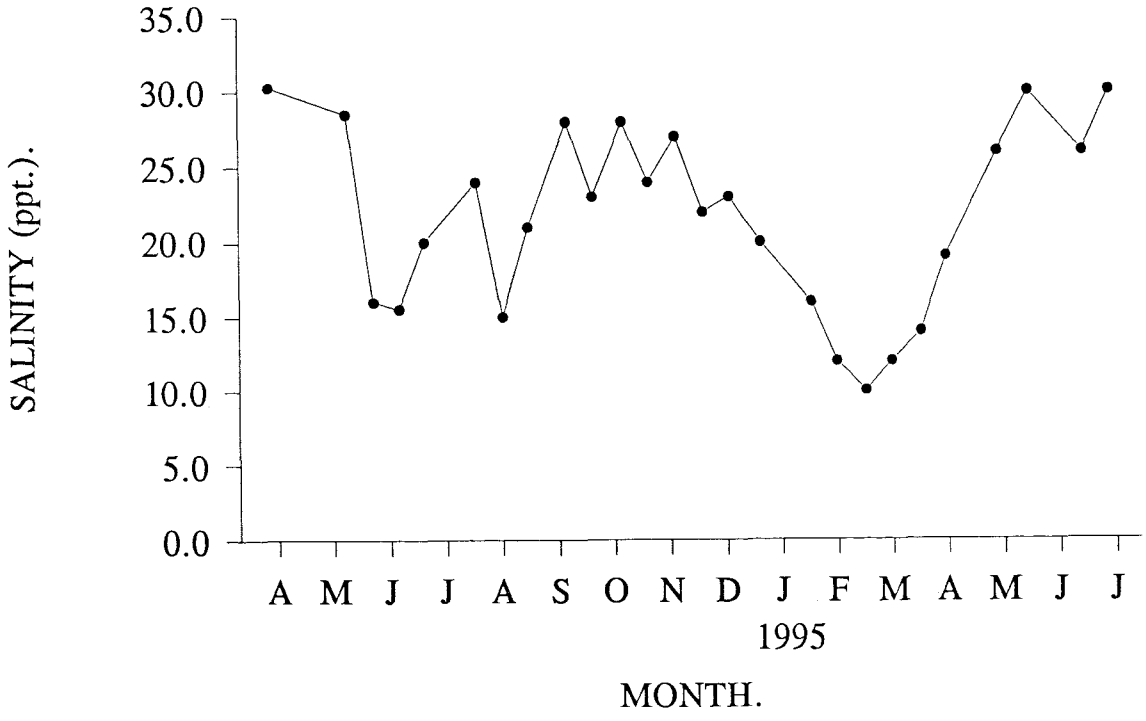

FIGURE 3.04 Variation in the salinity of surface water for the River Itchen during 1994 and 1995.

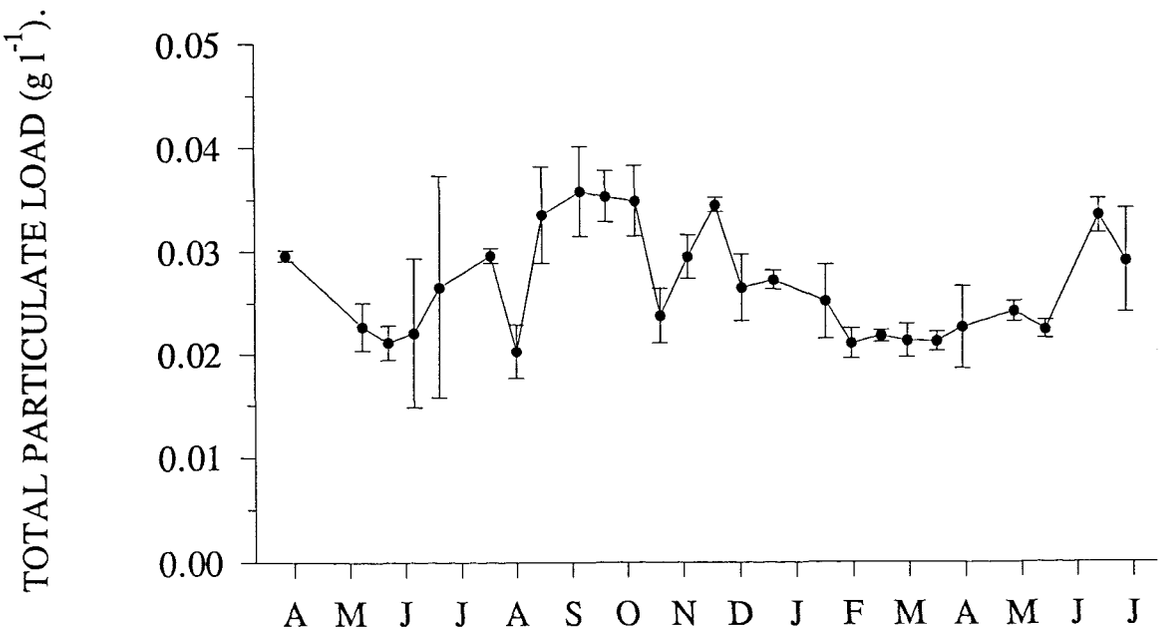

FIGURE 3.05 Seasonal variation in total particulate load for the River Itchen during 1994 and 1995 sowing the mean ± one standard deviation of 3 observations.

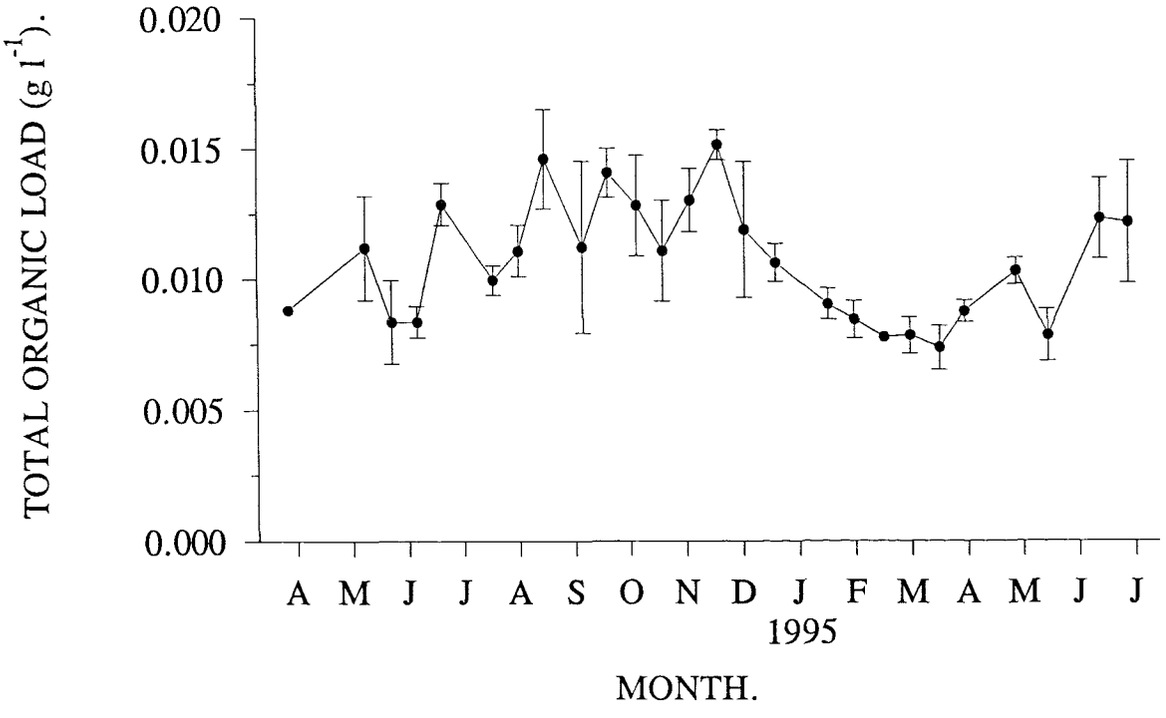

FIGURE 3.06 Variation in total organic load for the River Itchen during 1994 and 1995 showing the mean ± one SD of three observations.

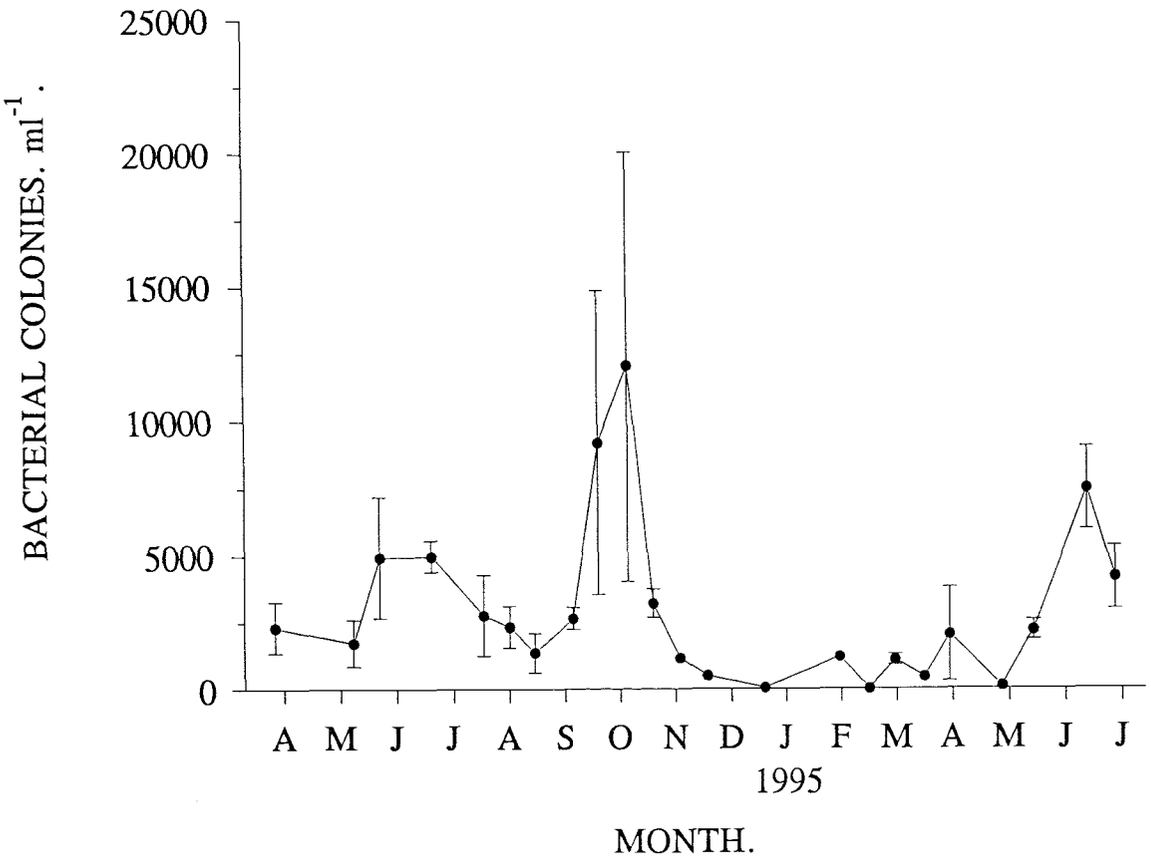

FIGURE 3.07 Variation in bacterial population (total viable count, TVC_{10}) for the River Itchen during 1994 and 1995 showing the mean ± one standard deviation of three observations.

respectively). As can be seen from table 3.01 there were positive correlations between the number of bacterial colonies present and the total particulate load, total organic load, temperature and salinity.

Preliminary attempts were made to determine the nature of the bacterial species found, the results of which have been summarised in table 3.02. Although not formally recorded, the diversity of colony types was generally low with colony type 3 being dominant except during the period June to October when all five colony types regularly appeared.

3.3.2 INDICES OF ORGANISM CONDITION.

All of the crabs collected *and* sampled during this study proved to be males (pers. obs.). No females of sufficient size to bleed were found in the trap, hence removing any consideration of sex linked differences in metabolism and immunocompetence.

3.3.2.1 METABOLIC INDICES.

The data collected relating to the metabolic status of *C. maenas* during the year have been summarised in figures 3.08, 3.09a and 3.09b. Figure 3.08 summarises the variation in haemolymph total sugar concentration as well as haemolymph glucose and trehalose. The glucose and trehalose records are incomplete as problems with developing the assays prevented their use initially (see section 3.2.2.3). Figure 3.09a summarises the monthly variation in haemolymph hydrogen peroxide and figure 3.09b indicates the mean carapace width of the sampled crabs showing the three sampling dates when the mean size of crabs was significantly lower than for the rest of the fifteen months. This data was removed from further statistical analysis as has been previously discussed in section 3.2.4.

Figure 3.08 indicates that all of the sugar data was variable emphasising the high level of individual variation amongst sampled crabs. The mean concentration of total anthrone sugars tended to be higher in mid summer (June) with the mean

COLONY	APPEARANCE ON GMA PLATE	GRAM STAIN REACTION AND APPEARNCE	TCBS AGAR REACTION	LAM AGAR REACTION
1	"SKIN" COLOUR	- VE RODS	+ VE	NOT TESTED
2	PINK	+ VE COCCI	?	NOT TESTED
3	CREAM/WHITE	- VE RODS	+ VE	NOT TESTED
4	SHINY GREY	- VE RODS	+ VE	NOT TESTED
5	ORANGE/YELLOW	+ VE COCCI?	- VE	NOT TESTED
INFECTED HAEMOLYMPH BACTERIA	CREAM/WHITE	- VE RODS	+ VE	+ VE

TABLE 3.02 Sumarising the preliminary identification of the bacterial species present in the seawater samples and also the bacteria collected from the haemolymph of an infected crab. Usually only one colony type (number 3) was found growing on the agar but during the summer months all five colony types were found. The seawater colonies were not plated on LAM agar as the method had not been developed at the time. Discussed more completely in section 3.3.1.

- VE = negative reaction, + VE = positive reaction, ? = results inconclusive.

sugar concentration on 6th June 1994 of 50.67 ± (SD) 9.88mg. per one hundred ml. proving significantly greater than the lowest sugar concentration of 18.35 ± 12.19mg. per one hundred ml. recorded on 31st March 1995. As indicated in table 3.01 there was a significant positive correlation between the sugar concentration and the total particulate load and a significant negative correlation between sugar and bacterial load.

It can be seen from table 3.01 that the concentration of circulating glucose positively correlates with the total sugar concentration. The data for glucose are again very variable although as before the mean concentration was at its highest during late summer (44.72 ± 15.62mg. per one hundred ml. on 1st August 1994) subsequently declining through the winter to reach a minimum during March and April (11.09 ± 3.18mg. per one hundred ml. on 31st March 1995). The glucose concentration on 1st August 1994 proved to be significantly higher than that on 31st March 1995 (Kruskal-Wallis ANOVA, $P < 0.05$). Table 3.01 also shows that there was a significant positive correlation between haemolymph glucose and total organic load and water temperature.

The concentration of trehalose in the sampled crabs positively correlated with all other measured parameters that were tested, except glucose (see table 3.01), although this may well have been a function of the limited data set available for test. Perhaps most interestingly, the concentration of trehalose correlated with the concentration of circulating hydrogen peroxide (correlation coefficient = 0.349, $P < 0.05$). None of the measured trehalose concentrations proved significantly different (ANOVA, $P > 0.05$; excluding the possibly size related data) and as before the data proved quite variable.

Haemolymph hydrogen peroxide concentrations fluctuated wildly throughout the year as shown in figure 3.09a and failed to correlate with any environmental variable measured. There was however a correlation between hydrogen peroxide and trehalose as shown in table 3.01 and mentioned above. The maximum recorded hydrogen peroxide concentration was 31.71 ± 25.27mg. per litre (5th September 1994) whilst the minimum was found in October 1994 (4.33 ± 2.22mg. per litre).

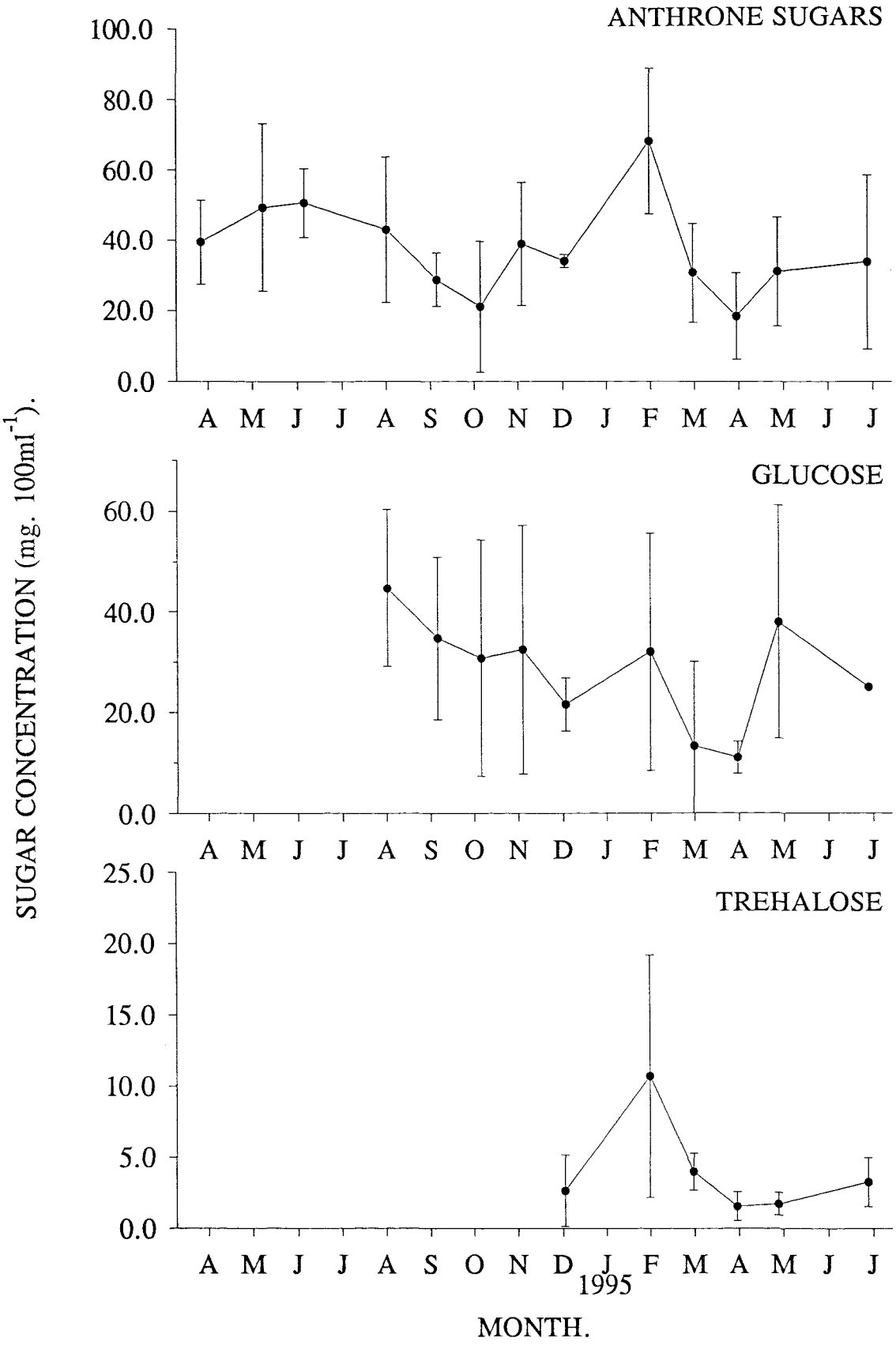

FIGURE 3.08 Variation in haemolymph sugar concentrations in the sampled crabs.

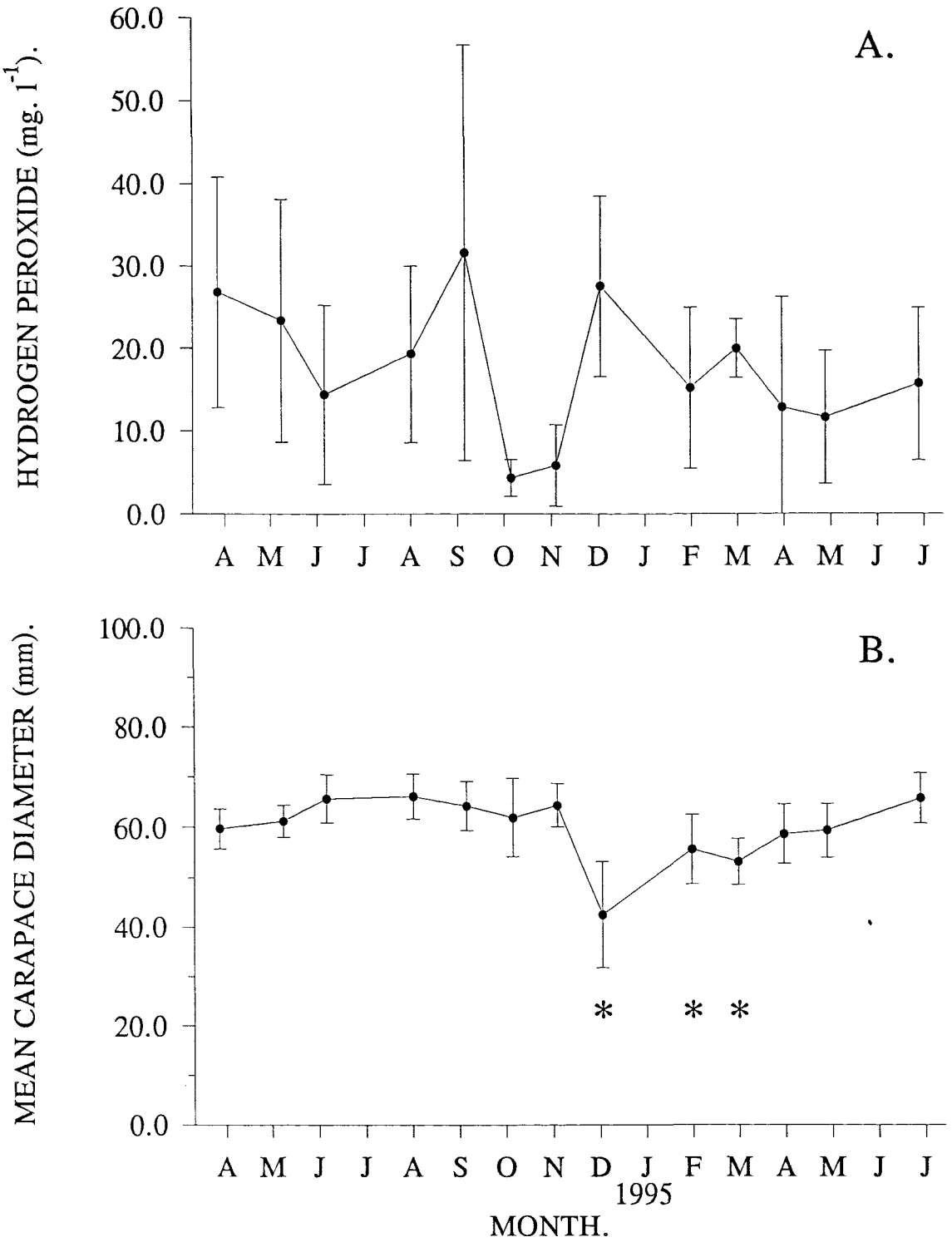

FIGURE 3.09 a. Variation in haemolymph hydrogen peroxide concentrations in the sampled crabs. Figure shows the mean ± one standard deviation. b. Mean size of sampled crabs for figures 3.08 and 3.09 showing the three occasions marked with an asterisk when the mean size was significantly smaller than for the rest of the year.

3.3.2.2 INDICES OF IMMUNOCOMPETENCE.

Figures 3.10a and 3.10b summarise the seasonal variation in the two cytological measures of immunocompetence: total haemocyte count (figure 3.10a) and granulocyte: hyalinocyte ratio (figure 3.10b). As before, variation in the mean size of sampled crabs has been presented in figure 3.10c, the asterisks again marking those occasions when the sampled crabs were significantly smaller.

The measures of haemocyte population are very variable emphasising the high degree of individual variation in *C. maenas*. There were however significant differences between the haemocyte population on 13th June 1995 (49.39 $\pm$ 12.00 x 10^6 cells per ml.) and 20th June 1994 (11.19 $\pm$ 6.26 x 10^6 cells per ml.) and between 15th May 1995 (48.00 $\pm$ 22.77 x 10^6 cells per ml.) and 20th June 1994. Table 3.01 indicates that in spite of this variability in the haemocyte data, there were significant positive correlations between haemocyte count and salinity and haemocyte count and granulocyte: hyalinocyte ratio.

Extreme individual variation was also evident in the granulocyte: hyalinocyte ratio and, as with the haemocyte counts, there was no obvious trend associated with the seasonal cycle. Table 3.01 indicates that there were positive correlations between granulocyte: hyalinocyte ratio and bacteria, total particulate load and, as discussed above, total haemocyte count. The maximum granulocyte: hyalinocyte ratio recorded in this study was 3.70 $\pm$ 0.87 on 23rd May 1994 (ignoring possible size related effects) whilst the lowest ratio of 2.09 $\pm$ 0.44 was recorded on 19th October 1994.

As discussed in chapter 2 (section 2.3.1.4) phenoloxidase activity in the cacodylate treated controls was always very low and, rather than discuss both the trypsin treated and control HLS activities in turn, the control activity has simply been subtracted from the trypsin treated HLS activity. It is evident from figure 3.11a that the phenoloxidase enzyme activity demonstrated extreme variability throughout the period of this study. Figure 3.11b indicates the mean carapace width of the sampled crabs and shows the two occasions when the crabs were significantly smaller than for the rest of the study. From figure 3.11a it can be

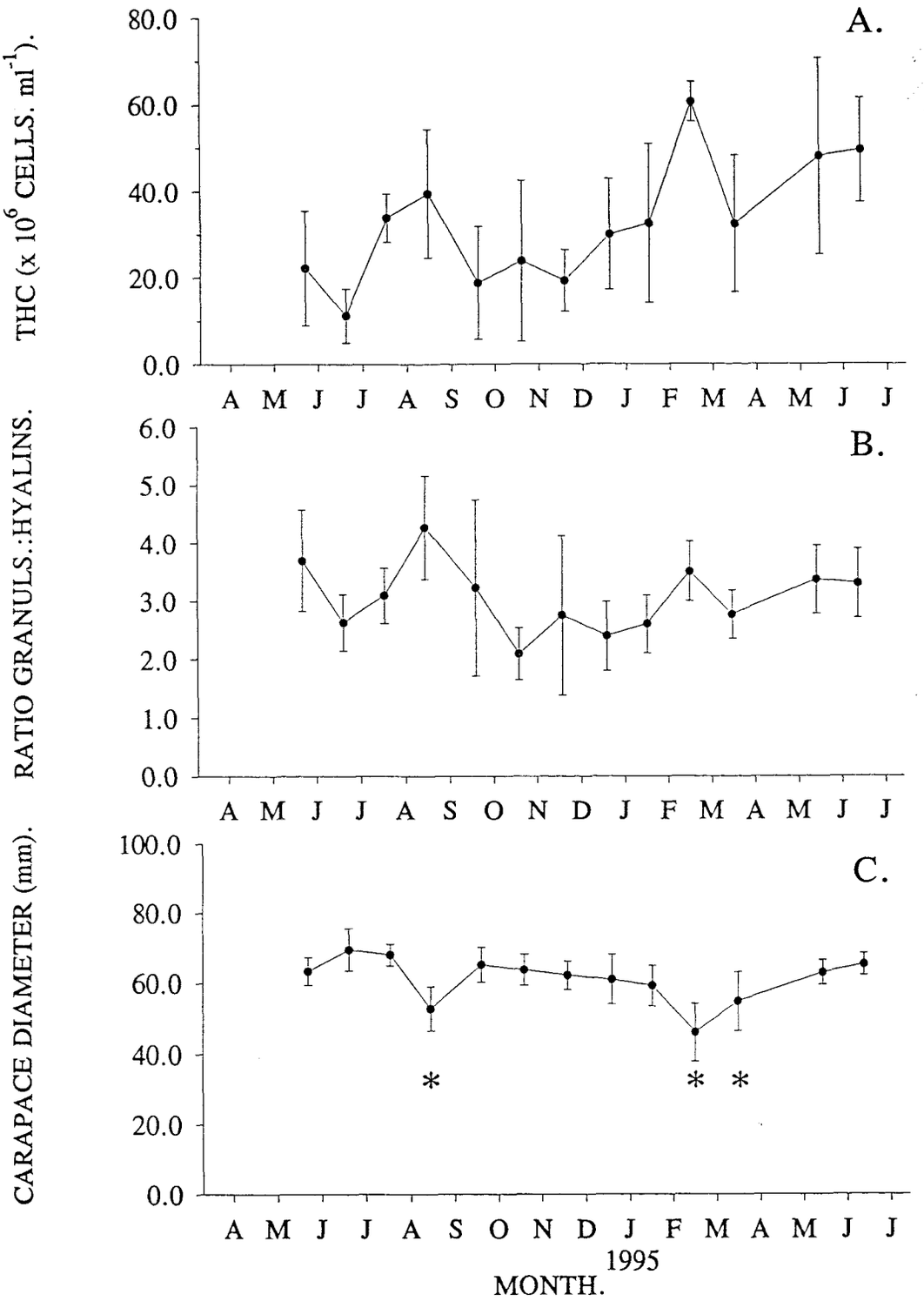

FIGURE 3.10 a. Variation in total haemocyte count for the sampled crabs. Figure shows the mean ± one standard deviation. b. Variation in the granulocyte: hyalinocyte ratio. c. Mean size of sampled crabs showing the three occasions when the mean size was significantly smaller than for the rest of the year (marked with an asterisk).

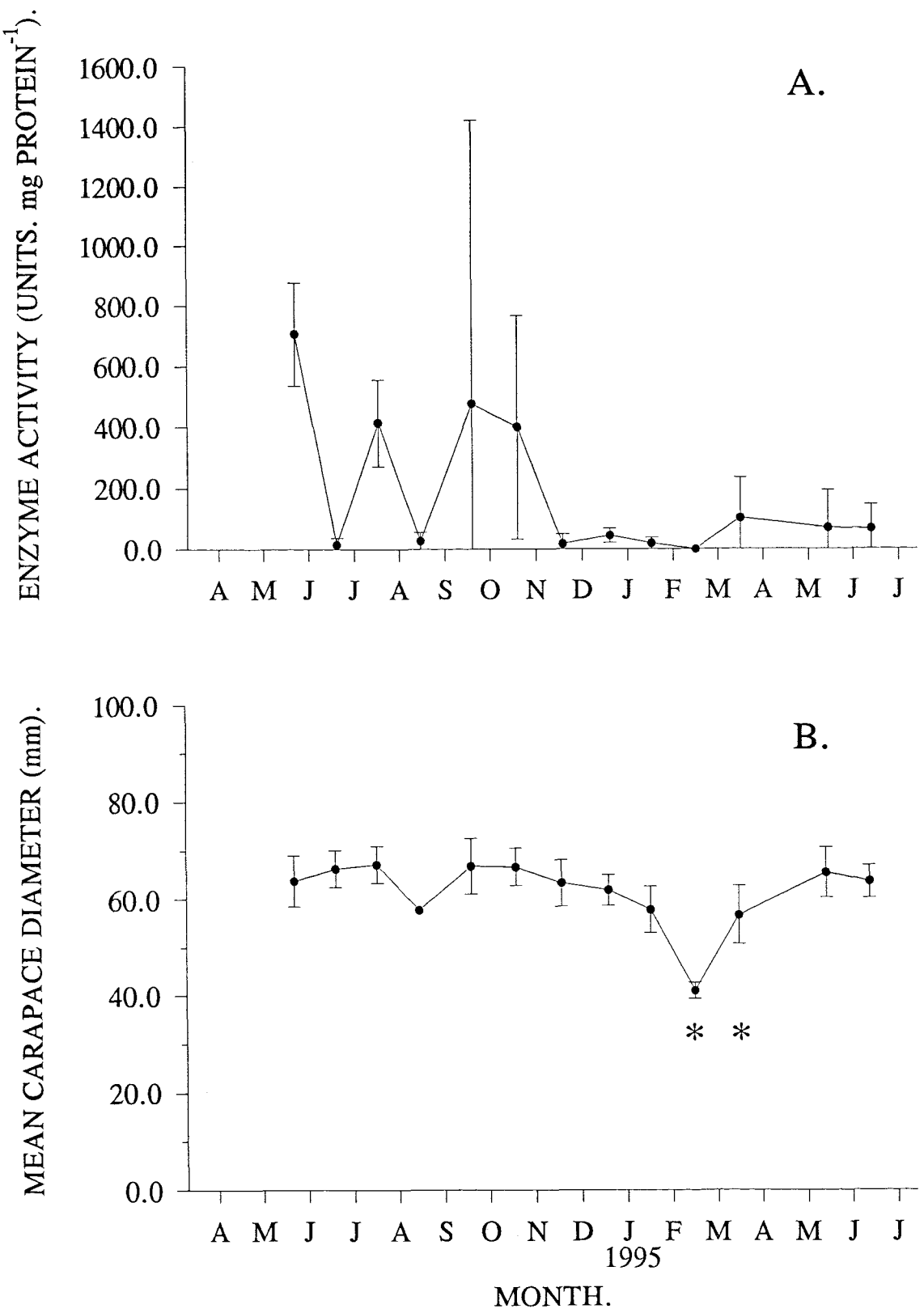

FIGURE 3.11 a. Variation in phenoloxidase enzyme activity for the sampled crabs. Figure shows the mean ± one standard deviation. b. Mean size of sampled crabs showing the two occasions when the mean size was significantly smaller than for the rest of the year marked with an asterisk.

seen that there were no obvious trends in the activity of the phenoloxidase enzyme, although there tended to be very little activity during the first few months of 1995. The highest and most variable enzyme activity was recorded during the months of September and October 1994 (476.18 $\pm$ 947.13 and 400.42 $\pm$ 367.52 units per mg. of protein respectively) which significantly correlated with the highest bacterial densities (table 3.01, $P < 0.05$).

3.4 DISCUSSION.

This chapter represents the first comprehensive investigation of the changes in metabolic status and immunocompetence of *C. maenas* subjected to *in situ* natural perturbation associated with the seasonal cycle. Further to this aspects of the changing physicochemical and biological nature of the environment have also been recorded in an attempt to correlate changes in the status of *C. maenas* with the seasonal cycle.

3.4.1 ENVIRONMENTAL PARAMETERS.

The temperature fluctuations measured in this study (20.0°C to 6.0°C) are comparable with previous studies in the Solent region (Hawkins & Hutchinson, 1990; and Brooks, 1994) although the salinity changes recorded here (30.3‰ to 10.0‰) fluctuate more widely than those reported by earlier workers. This can be explained by the fact that the earlier work of Hawkins & Hutchinson (1990) and Brooks (1994) was undertaken near Sowley, an area further out to sea than the sampling site used in this present study. Inputs to the site of trap deployment used in this investigation were dominated by the River Itchen which at its source would have been essentially freshwater. The low salinity recorded during January, February and March 1995 is likely to have been a function of increased precipitation and an increase in the volume transport of the River Itchen; whilst the high salinity evident in the summer would have been a function of reduced precipitation, increased evaporation and a reduction in the transport of the River Itchen which may have led to more saline water intruding into the higher reaches of Southampton Water.

The resultant particulate and organic load of the water column measured in this present study would have been caused by a number of different processes operating simultaneously. Needless to say an important source of suspended load would have been the River Itchen draining local land, but also some of the particulates present in the water column would have originated from the benthic environment following high wind events and the associated turbulence produced.

An increase in the organic load during the late summer and autumn of 1994 could also have been attributable to a local phytoplankton bloom occurring in the upper reaches of Southampton Water or the breakdown products of a senescent summer bloom (Raymont & Carrie, 1964; Savage, 1965; Bryan, 1979; and Antai, 1990). The high salinity at this time suggests a reduction in river input and the possibility that seawater may have extended further up the estuary bringing with it marine phytoplankton species and any senescent phytoplankton from a summer bloom.

This concept of senescent and dead plankton moving up the estuary correlates with the data collected pertaining to bacterial abundance. Table 3.01 indicates that the bacterial density positively correlated with organic load, a proportion of which could have been due to dead and decaying organic material possibly from a bloom. Fuhrman (1980) and Antai (1990) have both reported that estuarine bacterial production is dependent on the leakage of dissolved organic matter following the ageing and disruption of phytoplankton at the end of a bloom. Table 3.01 also shows that the bacterial density recorded in this study correlated with the total particulates. Indeed, Paerl (1974) has reported that bacteria are usually found attached to particles in shallow coastal waters and it is therefore conceivable that the increase in bacterial population during September and October 1994 was caused by the resuspension of particulate associated bacteria during high wind events. This theory may also go some way to explain the patchy or contagious, and hence variable, densities found during these months. As highlighted in the results (section 3.3.1), bacterial densities also correlated with water temperature, an observation which is supported by the work of Antai (1990). It can be postulated that as the temperature rose the water became more suitable for a wider range of species and indeed the observations made relating to the bacterial diversity would suggest that there were more species of bacteria present during the warmer summer months.

The peak in bacterial abundance during September and October 1994 represents an increase of approximately an order of magnitude over the winter densities, a value supported by earlier work (Antai, 1990). The measured density of bacterial colonies in the present study (in the region of 10^6 bacterial colonies per litre) is considerably lower than the densities reported by other workers in

previous studies. For example: Goulder (1977) has reported values in the region of 10^9 bacteria per litre for the Humber estuary and Linley *et al.* (1983), working in the English Channel, have recorded values of 10^8 per litre. These differences by orders of magnitude may have been a function of the different sites chosen for study and the different conditions associated with each site; or they may simply be an effect of the different methodologies adopted. Previous workers have used methods which count individual bacterial cells whilst, the plating method used in this present study represents more of an estimation as a number of bacteria on a plate may combine to form a single colony developing on the agar. This less accurate method was adopted in this present study as the trends in abundance were of more interest rather than the absolute number of bacteria present and the plating method was a more rapid means of visualising bacterial population than the cell count methods of Goulder (1977) and Linley *et al.* (1983).

Analysis of the bacteria from the grossly infected haemolymph of a small percentage ($< 5\%$) of sampled crabs proved that the infectious agent was a strain of the species *Listonella (=Vibrio) anguillarum*. The potential occurrence of strains of this species has been demonstrated in the past as reviewed by Johnson (1983) and Chisholm (1993). Further to this *Listonella anguillarum* has been shown to have a widespread marine and clinical distribution (Anderson & Conroy, 1970; and Farmer *et al.*, 1985).

It is interesting to note that only a small percentage of sampled crabs had any gross infection evidenced by a milky white haemolymph. Indeed it would seem that lethal infection is very rare in the natural environment in spite of all of the perturbations discussed above. Sindermann & Lightner (1988) have comprehensively reviewed disease in marine aquaculture and it is evident from their work that most of the documented infection occurs following wounding in crustaceans held at high density, such as in crab shedding tanks. It is therefore conceivable that animals at a "low density" in the wild rarely become infected because of the low incidence of wounding. On entering a baited pot they may have been more susceptible to wounding due to the increased density of animals competing for a single food item. The studies of Dillaman & Roer (1980) and Halcrow & Smith (1986) indicate that wound closure in *C. maenas* is not an

immediate process and whilst open any hole in the carapace would provide a portal of entry for the bacteria in the water column. It can be concluded that the incidence of disease in wild populations of *C. maenas* collected from Southampton Water is minimal and any incidents of infection found in this present study may well have been a function of the method of capture.

3.4.2 METABOLIC INDICES OF ORGANISM CONDITION.

The results in section 3.3.2 show that the crabs collected in the pot were dominated by males with very few of the crabs caught being female (< 2%). This has been observed before by Warman *et al.* (1993) who investigated tidal migratory behaviour in the shore crab, *Carcinus maenas*. These workers demonstrated that a high proportion of females avoided the intertidal and shallow sublittoral and foraged less intensively than the males. Rasmussen (1959) had earlier indicated that ovigerous females move offshore whilst the larvae hatch to avoid reduced salinity. It would seem therefore that females are behaviourally adapted to avoid areas where the daily and seasonally associated salinity fluctuations are great.

McGaw & Naylor (1992) and Warman *et al.* (1993) have both also suggested that salinity tolerance differs between the red and green colour morphs of the shore crab, with green crabs being more salinity tolerant and dominating the intertidal zone and the red colour morphs being more prevalent in the subtidal. Initially it was hoped to investigate the metabolic and immunological differences between the two colour morphs although this turned out to be unfeasible as not enough crabs of either colour were sampled in any one month to allow meaningful statistical comparisons to be performed. Further to this the data from repeated sampling events could not be combined without interference by seasonal and size related effects.

From the present investigation the high degree of individual variability is apparent as has been previously discussed by Thabrew *et al.* (1971). With such a large amount of variation between individuals statistical comparisons have in most

instances proved inconclusive. Having said this some correlations tested did prove significant ($P < 0.05$) as summarised in table 3.01. It must be pointed out however, that even though correlations between variables have been shown to exist, "cause and effect," is not necessarily implied. Had it been possible to determine which independent variables were influencing the indices of metabolic status and immunocompetence regression analysis would have proved to be a more suitable and stringent test.

Table 3.01 does show that there was a significant positive correlation between haemolymph glucose and water temperature. This agrees with the observations of Dean & Vernberg (1965) on the crabs *Uca pugilator* and *U. minax* but disagrees with the work of Thabrew *et al.* (1971) on *C. maenas*. It must be pointed out however, that Thabrew *et al.* (1971) reported data for isolated gill tissue; Heath & Barnes (1970) who investigated the hepatopancreas of *C. maenas* found there to be no seasonal change in the carbohydrate composition. It would seem therefore that carbohydrate metabolism may be tissue specific - an idea which has been suggested by Hohnke & Scheer (1970).

As mentioned in the results the concentration of anthrone sugars in the haemolymph of *C. maenas* correlates with both the glucose and trehalose concentrations. This result is perhaps expected as both glucose and trehalose are constituents of the total reducing sugar pool. In this instance however there is no correlation, positive or negative, between the anthrone sugar concentration and haemolymph hydrogen peroxide as was demonstrated in chapter two (section 2.3.1.2). This observation may be due to the large number of variables acting simultaneously in this present study but would suggest that oxidative metabolism cannot solely explain the generation of hydrogen peroxide as an end product. It is possible that some of the hydrogen peroxide measured is due to the formation of superoxide ions as a non specific immune defence. This idea is developed further below.

The correlations between anthrone sugars and particulate load and bacterial population are more difficult to interpret. It is possible that the negative correlation between sugar concentration and bacterial density may have been the result of the utilisation of sugars in those metabolic processes associated with immune defence.

Smith & Chisholm (1992) have reviewed the occurrence and role of crustacean agglutinins which promote the aggregation of foreign particles. Enhanced titres of agglutinins have been reported by Pauley (1973) and Adams (1991) to be inducible following exposure of the host (*Callinectes sapidus* and *Penaeus monodon* respectively) to suitable test particles and it is possible that such induction requires oxidative metabolic processes using sugar substrates. Similarly, the review by Söderhäll & Cerenius (1992) has shown that the process of phagocytosis which has been demonstrated in *C. maenas* undoubtedly requires energy. As indicated by Johnston & Spencer-Davies (1972) the measure of anthrone responsive sugars includes a glycogen component which is hydrolysed during the assay procedure. Glycogen is metabolised to the more metabolically available substrate glucose via the intermediate dimer trehalose (Feofilova, 1992) and in light of this it is interesting to note that the haemolymph trehalose concentration also positively correlates with bacterial abundance. It is therefore possible to postulate that, as the bacterial population increased, the immune processes requiring glucose caused a depletion of the glycogen reserves in the haemolymph (reducing the anthrone responsive sugar concentration) as they were catabolised to the glucose dimer trehalose which was more metabolically available.

These ideas do remain equivocal as the glucose data do not support any such conclusion since it failed to correlate with the bacterial population. Furthermore, as cautioned earlier, the negative correlation between bacteria and anthrone sugars may be coincidental. The bacterial data represent an external population, whilst the immune defence processes would be expected to respond to changes in the internal pathogen load.

As a consequence of this uncertainty the role of trehalose remains elusive. The limited trehalose record precludes any realistic discussion of seasonally associated variability and, indeed as discussed in section 3.3.2.1, the fact that the trehalose concentration correlated with all but one of the other variables tested is probably a function of the limited data set available. Schwoch (1972) investigated the role of trehalose in adult crayfish, *Orconectes limosus*, demonstrating that the disaccharide was synthesised at a greater rate during the months of February and March than in the summer and also that, following its conversion to glucose, the

trehalose was used for chitin biosynthesis.

Schwoch (1972) also found that the trehalose was rapidly converted to glucose within the haemolymph of *O. limosus* - an observation echoing the earlier work of Telford (1968). The other potential roles of trehalose demonstrated at a microbiological level have been more completely discussed in the introductory chapter (section 1.3.3) and it is not proposed to repeat them here, although one interesting correlation indicated in table 3.01 is however the relationship between haemolymph trehalose and hydrogen peroxide. One of the possible roles for trehalose in the fungi is to act as a "sponge" for roaming superoxide radicals (Feofilova, 1992) and this may also be occurring in *C. maenas*. The earlier *in vitro* work of Bell & Smith (1993) on *C. maenas* and Song & Hseih (1994) on the tiger shrimp, *Penaeus monodon*, discussed in chapter two have both suggested the possibility of superoxide production in crustaceans as a form of non specific immune defence. It is possible that the trehalose may be acting as a self defence against radicals produced by *C. maenas* in response to invading pathogens which may be present in crabs sampled from the field. If these radicals do not encounter the bacteria they are intended to destroy they may cause serious damage to the crab tissues and the trehalose may soak up any excesses producing hydrogen peroxide which can further be metabolised to less destructive compounds.

3.4.3 INDICES OF IMMUNOCOMPETENCE.

Haemocyte population and composition data show very little predictable variability associated with the seasonal cycle. There was however a significant positive correlation between the haemocyte count and salinity and a negative correlation between haemocyte population and organic load. As discussed by Schoffeniels & Gilles (1970) and Bauchau (1981) the haemocytes of *C. maenas* contain a pool of free amino acids which could play a significant role in osmotic regulation in the crab. *C. maenas* is a well known weak osmoregulator (Pequeux, 1994) able to control the osmolarity of its haemolymph to a limited extent. Mantel (1983) has however, demonstrated that as the osmolarity of the external medium rises above 750 milli-osmoles per kilogramme *Carcinus maenas* becomes an

osmoconformer and it is possible that the increase in haemocyte number, with its associated free amino acid pool, at high salinity is a function of the haemolymph osmolarity conforming to the external milieu.

What is most apparent from figure 3.10 is that the smaller animals sampled (those occasions marked with an asterisk) tended to have a higher cell count (quoted as cells per ml.) than the larger individuals. At the same time the smaller crabs also tended to have a higher granulocyte: hyalinocyte ratio than the larger crabs. These observations indicate that the smaller crabs had a greater proportion of granulocytes in circulation and suggests that as the animals grow, through a series of moults, the haemolymph volume increases with little change in the total number of haemocytes *per animal* but with an increase in the proportion of phagocytic hyalinocytes. This observation is supported by a significant positive correlation between the haemocyte density (cells per ml.) and the ratio of granulocytes to hyalinocytes (table 3.01) indicating that at higher cell densities (recorded in the smaller crabs) there was a greater proportion of granulocytes circulating.

Unlike the earlier work of Dean & Vernberg (1966), Ravindranath (1977), Truscott & White (1990), and Chisholm & Smith (1994) there was no apparent relationship between haemocyte number and temperature. This may well have been due to the large number of impinging variables operating in concert in this present study as has been previously suggested by Chisholm (1993). It would seem that the haemocyte population and composition is a complex function of a number of different environmental factors, all of which have not been sampled in any individual study.

As has been pointed out repeatedly the individual variation shown in the data collected was very large and this reached an extreme in the case of the phenoloxidase enzyme activity indicated in figure 3.11a. In spite of this variability there was a significant positive correlation between phenoloxidase enzyme activity and bacterial density as summarised in table 3.01. In chapter 2 (section 2.4) the measure of phenoloxidase enzyme activity was clarified and it was indicated that the activity really represented that enzyme that was "locked away", as the

zymogen prophenoloxidase, in the granulocytes at the time of bleeding. It is therefore apparent that as the bacterial population increased the reserves of this enzyme were built up suggesting a mechanism of immunostimulation. Sung *et al.* (1994) have demonstrated temporarily induced resistance to *Listonella* spp. in the tiger shrimp, *Penaeus monodon*, following inoculation of the host with β-glucans, concluding that this temporary immunity was due to the stimulation of the phenoloxidase pathway. β-glucans are components of bacterial cell walls and it is tempting to suggest that the present study may be the first report of a mechanism of immunostimulation in the shore crab, *C. maenas*. As cautioned earlier however, the correlation observed may well be coincidental as the measure of bacterial population is essentially an external measure whilst the variation in phenoloxidase enzyme activity would be expected to be a function of the internal pathogen load. Furthermore, as discussed in the previous chapter, it would have proved interesting to investigate the active *in vivo* enzyme component in the haemolymph at the time of bleeding, unfortunately a suitable assay protocol for measuring phenoloxidase enzyme activity in the plasma was not developed in sufficient time.

To conclude this discussion it is evident from this study that there are predictable relationships between a number of indices and environmental parameters. Not all of the correlations demonstrated in this study can be explained with the present level of understanding however, and as discussed some of the correlations may be coincidental. It must be remembered that the tidal cycle variability demonstrated in the previous chapter would be superimposed on the long term trends observed in this study and that both seasonal and tidal baseline variability must be considered when adopting biomarkers to monitor changes in water quality.

Having now defined tidal and seasonal associated baselines with these first two chapters the responses of *C. maenas* to artificially imposed stresses applied in a controlled environment can now be assessed.

CHAPTER FOUR

EFFECTS OF BACTERIAL CHALLENGE ON THE CONDITION OF *Carcinus maenas*

CHAPTER FOUR

EFFECTS OF BACTERIAL CHALLENGE ON THE CONDITION OF Carcinus maenas

4.1 INTRODUCTION.

Having previously identified variation in the condition of *Carcinus maenas* associated with tidal and seasonal cycles (chapters two and three) the effects of laboratory induced stress could now be investigated.

This chapter summarises the metabolic and immunological responses of *Carcinus maenas* to a non-lethal pathogenic infection using the Gram negative bacterium *Listonella* (= *Vibrio*) *anguillarum*. As has been determined in chapter three the choice of this bacteria is appropriate, as it has been found to occur naturally both in seawater samples collected from the River Itchen and within the milky white haemolymph of infected crabs collected from traps in the Itchen. In a wider context *L. anguillarum* is a well-documented species with both a marine and clinical distribution (Anderson & Conroy, 1970; and Farmer *et al.*, 1985) which, in many cases, has been demonstrated to directly impinge on attempts to harvest marine resources.

Listonella anguillarum is typically a fish pathogen that is renowned for causing "red disease" in eels (Sindermann, 1968; de la Cruz & Muroga, 1989), but it has also been isolated from moribund shrimp and crabs (Johnson, 1983). *L. anguillarum* has been shown to cause the death of juvenile lobsters under experimental conditions and has been implicated in the occasional high mortalities found amongst commercially held blue crabs, *Callinectes sapidus* (Johnson, 1983).

Five hundred and seventeen separate strains of *Listonella anguillarum* have been isolated from diseased fish including Atlantic salmon (*Salmo salar*), sea trout (*Salmo tritta*), pike (*Esox lucius*) and rainbow trout (*Oncorhynchus mykiss*) (Larsen et al., 1994). In the ayu (*Plecoglossus altivelis*) and the Japanese eel (*Anguilla japonica*) lethal infection with *Listonella anguillarum* results in tissue necrosis,

liquefaction and haemorrhaging following bacterial multiplication in the host (Miyazaki, 1987; de la Cruz & Muroga, 1989) whilst in the rainbow trout death occurs after degeneration and necrosis of the hepatocytes and the muscle fibres (Lamas *et al.*, 1994).

Johnson's (1983) review of decapod pathobiology - as well as recording the occurrence of *L. anguillarum* in crustaceans - also discusses the multitude of different infectious agents occurring in marine invertebrates including viruses, rickettsial infections, bacteria and fungi. His work deals, in essence, with the symptoms of the diseases, simple pathological life histories and geographic occurrence; an alternative and more functional approach has been adopted by other workers who have investigated the immunological responses of decapods to infection with both denatured and live bacteria.

Smith & Ratcliffe (1980a & 1980b) have used heat killed *Bacillus cereus* and *Moraxella* spp. to investigate the histopathological responses of *Carcinus maenas* whilst White & Ratcliffe (1982) have used radio- and fluorescent labelled *Moraxella* spp. to investigate bacterial clearance and sequestration in C. *maenas*. More recently Martin *et al.* (1993) have used a range of species to investigate bacterial clearance in the penaeid shrimp, *Sicyonia ingentis*.

This study represents the first occasion on which the response of *C. maenas* to infection with *Listonella anguillarum*, a prevalent member of the marine microbial community, has been investigated. Laboratory based infections were studied so that other potential pathogens could be excluded and the effects of influences such as the tidal state or time of day could be removed.

4.2 METHODS.

4.2.1 COLLECTION, INFECTION AND GENERAL EXPERIMENTAL PROCEDURE.

Specimens of *C. maenas* were collected from the littoral zone using baited pots deployed on the salt marsh system around Hayling Island, Portsmouth (50° 50.1'N, 00° 58.6'W; see figure 1.02). In common with all experiments in this thesis only healthy intermoult male crabs were used (see section 2.2.1) and once collected individuals were allowed to acclimate, in the dark, to aquarium conditions of 19.6 ± (SD) 0.9°C, 35.5 ± (SD) 1.4‰ for two weeks.

During the acclimation period the animals were fed every other day on a diet of mixed fish but all crabs were starved for twenty-four hours prior to infection with bacteria and throughout the week incubation.

This investigation ran over a six week period starting on 24th November 1994. In the first week (7 day incubation) the changes produced in the circulating haemocyte population were monitored, followed by the metabolic studies in the second week. In the third week the response of the phenoloxidase enzyme system to pathogenic infection was monitored. All of the assay procedures were then repeated in a second three week study to maximise the size of the collected data set. In each of the week incubations a minimum of twelve organisms were incubated (six control, six experimental). For the experimental incubation crabs were maintained in a constant red light environment (Taylor & Naylor, 1977; Bolt *et al.*, 1989) in separate aerated chambers, each containing approximately one litre of sterile seawater (sterilised by autoclaving at 120°C for twenty minutes) which was replaced every two days (Smith & Ratcliffe, 1980a).

Crabs were transferred from the aquarium holding tanks to the infection chambers twenty-four hours prior to infection to allow them opportunity to adjust to the new environment.

To ensure that the crabs were infected with an actively dividing culture rather than one which was senescent, the growth of bacteria in culture was first

characterised as a separate study.

General marine broth (see appendix C.1) was inoculated with *Listonella anguillarum*, strain 3.72, donated by the Fish Diseases Laboratory, MAFF, Weymouth; and allowed to grow at 18 ± 1°C within an incubator. Every twelve hours following inoculation culture density was assessed using the fluorescent method described by Brooks (1994) and outlined in appendix C.2. Table 4.01 and figure 4.01 summarise the results of this preliminary study from which it was determined that thirty hour cultures of *Listonella anguillarum* would be most suitable.

CULTURE	CULTURE DENSITY (x 10^6 CELLS ml^{-1})			
	12 HOURS	24 HOURS	36 HOURS	48 HOURS
1	2.304	11.310	23.249	26.390
2	5.865	7.121	25.343	27.438
3	5.027	1.885	22.830	18.641
4	3.142	10.682	17.175	19.269
MEAN	4.084	7.750	22.149	22.934
SD.	1.645	4.323	3.494	4.622

TABLE 4.01 Bacterial growth in culture medium at 18 ± 1°C over a forty-eight hour period.

Initial studies aimed to determine a suitable sub-lethal dose of the bacterium *Listonella anguillarum* for a week (seven day) incubation. In contrast to the present work in which live cultures were used, earlier *in vivo* studies have either used a 0.1ml. injection of a solution of 10^9 cells.ml.$^{-1}$ of denatured bacteria (Smith & Ratcliffe, 1980a and 1980b) or have used different species of bacteria including: the Gram negative *Bacillus cereus* and the Gram positive *Moraxella* spp. (White &

Ratcliffe, 1982; White *et al.*, 1985); and *Bacillus subtilis*, *Pseudomonas flourescens*, *Listonella alginolyticus* and *Aerococcus viridans* by Martin *et al.* (1993). Culture density was assessed as described above and appropriate dilutions of the *Listonella* cultures were made by centrifuging the bacterial broth at 450*g* for fifteen minutes and diluting the sedimented pellet of bacteria with sterile *Carcinus* saline (Smith and Ratcliffe, 1978; see appendix A.5).

Crabs were infected by injecting 0.1ml of diluted bacterial broth into the fourth pereiopod via the unsclerotised membrane using sterile syringes and 25 gauge needles. Control crabs were sham injected with 0.1ml of sterile *Carcinus* saline (Smith & Ratcliffe, 1980b). Prior to infection the injection site was sterilised by cleaning with a solution of 70% ethanol (Smith & Ratcliffe, 1980a). Following infection the crabs were maintained in their incubation chambers for a week during which time any mortalities were recorded.

From the sub lethal studies a dose of 0.1ml of 10^5 bacteria ml^{-1} was found to be at the limit of lethality for a week incubation and consequently all future infections used such a dose.

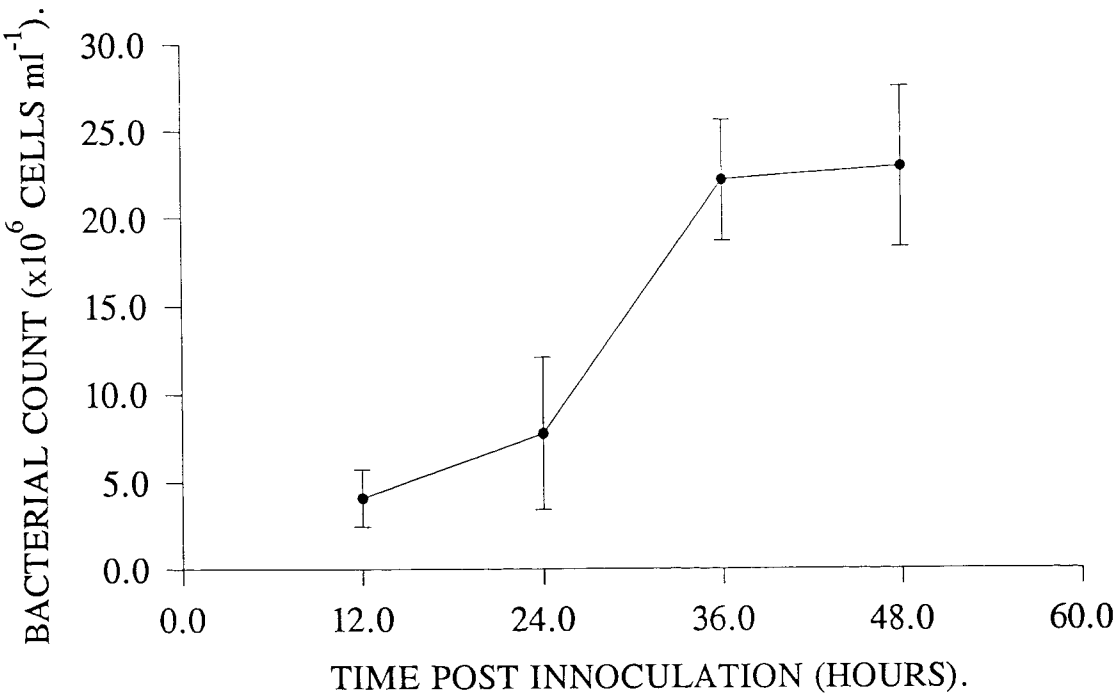

FIGURE 4.01 Growth of *Listonella anguillarum* in general marine broth at 18 ± 1 °C over a forty-eight hour period. Figure shows the mean and standard deviations as summarised in table 4.01. See text for full explanation.

4.2.2 BIOCHEMICAL AND IMMUNOLOGICAL METHODS.

All of the biochemical and immunological parameters were assayed as described in section 3.2.2 (page 75 to page 80).

4.2.3 HISTOLOGICAL EXAMINATION OF GILL TISSUE.

As demonstrated by Smith & Ratcliffe (1980a and 1980b) foreign particles, including bacteria, are removed from the circulation and are sequestered within the gill filaments in *C. maenas*. To verify these earlier observations crabs were sacrificed after a week incubation using a lethal injection (2-4ml) of a solution of 20% seawater formaldehyde and the gills were immediately excised and fixed in Davidson's seawater fixative (Bucke, 1989) before being dehydrated in an ethanol series and infiltrated with "Histowax Embedding Medium™". Sections of the gill lamellae (5μm.) were prepared using a Glen Creston™ microtome and stained with haematoxylin and eosin according to Bucke (1989) and then viewed using the bright field optics of an Zeiss™ KF2 microscope.

4.2.4 STATISTICAL ANALYSIS OF DATA.

Before any statistical analyses were performed all the data were tested for normality.

In those cases where the data were normally distributed *t* tests were used to analyse the differences between treatments (control and infected) after one day and after one week. Differences within a single treatment (ie. control *or* infected) over the week incubation were also analysed using *t* tests.

In those instances where the assumptions of parametric tests were violated the less stringent, non parametric, Mann-Whitney Rank Sum test was used as an alternative to the *t* test. In both cases the statistical significance tests were at the $P < 0.05$ level.

4.3 RESULTS.

4.3.1 HAEMOLYMPH SUGAR CONCENTRATIONS.

Changes in the composition and total concentration of the haemolymph sugars have been summarised in table 4.02 and figure 4.02. The data presented as "other sugars" has been inferred from the difference between the measured anthrone sugar concentration and the combined glucose and trehalose concentrations.

It can be seen from figure 4.02 that for both the control and infected crabs there was a decrease (of approximately 32% and 15% respectively) in total haemolymph sugars over the week incubation from mean values of 59.70 and 52.88 mg. per one hundred ml. after one day to 40.59 and 44.97 mg. per one hundred ml. after one week respectively. It was also evident that this decrease was mainly attributable to a 42% drop in the glucose concentration from approximately 45 mg. per one hundred ml. one day after infection to 26 mg. per one hundred ml. a week after infection.

From table 4.02 it is clear that the concentration of "other sugars" circulating within the haemolymph increased over the week long incubation, but also that the percentage contribution made to the total sugar pool by "other sugars" increased at the expense of the contribution made by glucose.

In the case of the control animals there was no real change in the concentration of the disaccharide sugar trehalose either in terms of concentration or percent contribution. For the infected individuals there was an increase in the percent contribution from 4.07% to 20.72% of total circulating sugar. It must be pointed out however, that statistical analysis of the data proved all changes outlined above to be non significant ($P > 0.05$).

Figure 4.03 resolves figure 4.02 into the separate constituent sugars and indicates the large standard deviations of the collected data emphasising a high level of inter-individual variability.

+ 1 DAY.						
SUGAR	CONTROL			INFECTED		
	mg. $100ml^{-1}$	%	N	mg. $100ml^{-1}$	%	N
TOTAL SUGAR	59.70 ± 34.42	100.00	9	52.89 ± 22.42	100.00	12
GLUCOSE	48.20 ± 26.13	80.73	10	42.98 ± 13.40	81.26	12
TREHALOSE	7.95 ± 7.41	13.31	8	2.15 ± 3.84	4.07	12
OTHER SUGARS	**3.55**	**5.96**	-	**7.76**	**14.67**	-

+ 1 WEEK.						
SUGAR	CONTROL			INFECTED		
	mg. $100ml^{-1}$	%	N	mg. $100ml^{-1}$	%	N
TOTAL SUGAR	40.59 ± 14.84	100.00	6	44.97 ± 19.93	100.00	10
GLUCOSE	25.97 ± 25.69	63.98	7	26.90 ± 27.58	59.81	11
TREHALOSE	5.49 ± 3.81	13.52	6	9.32 ± 8.51	20.72	9
OTHER SUGARS	**9.13**	**22.50**	-	**8.75**	**19.47**	-

TABLE 4.02 Changes in the haemolymph sugar composition of *Carcinus maenas* following inoculation with *Listonella anguillarum* and a subsequent week incubation. Table shows means ± one standard deviation of N observations. Values in bold have been inferred as discussed in the text.

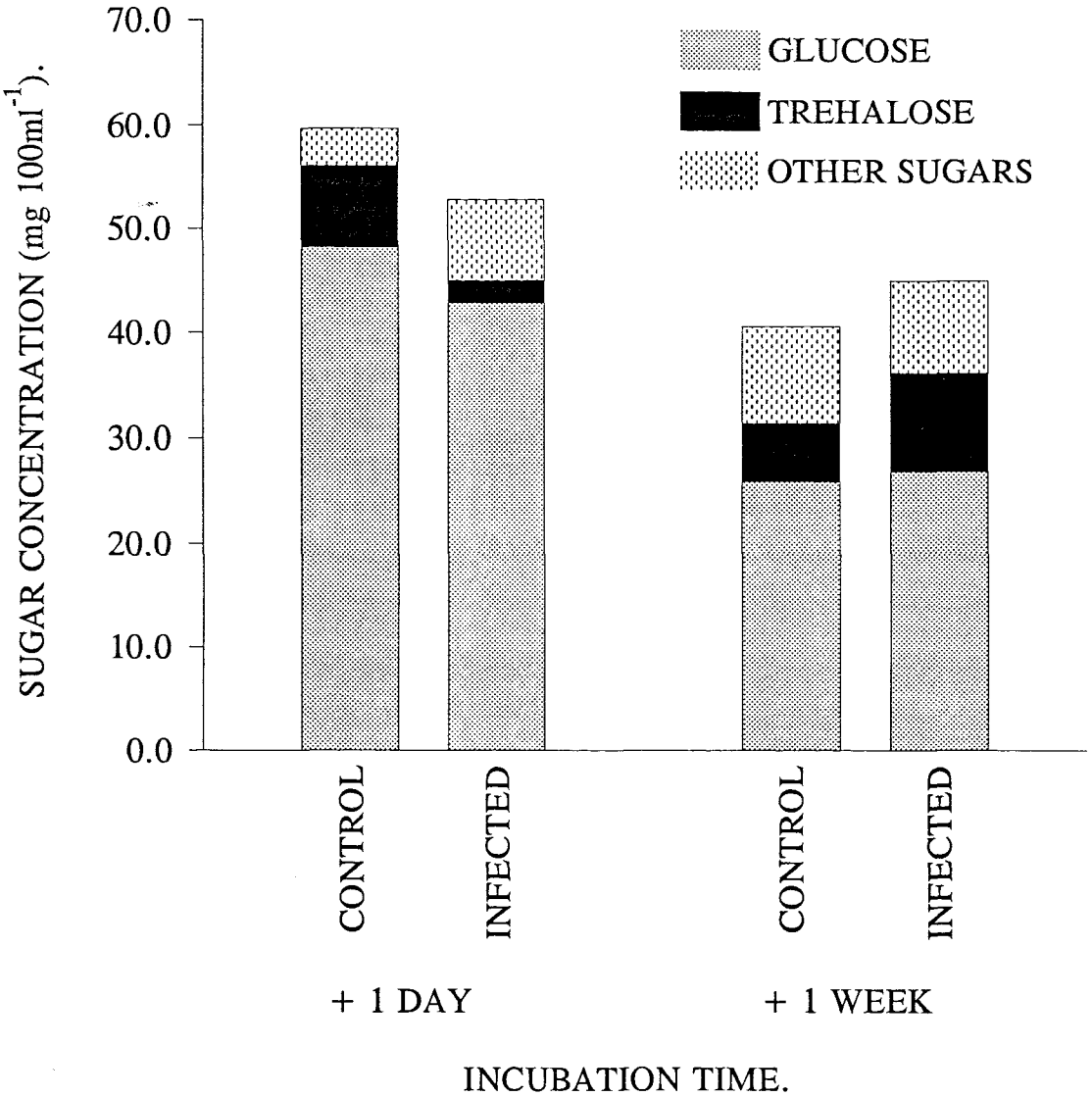

FIGURE 4.02 Changes in haemolymph sugar composition in infected and control animals following a week incubation. Figure shows mean values and "other sugars" have been inferred as discussed in the text.

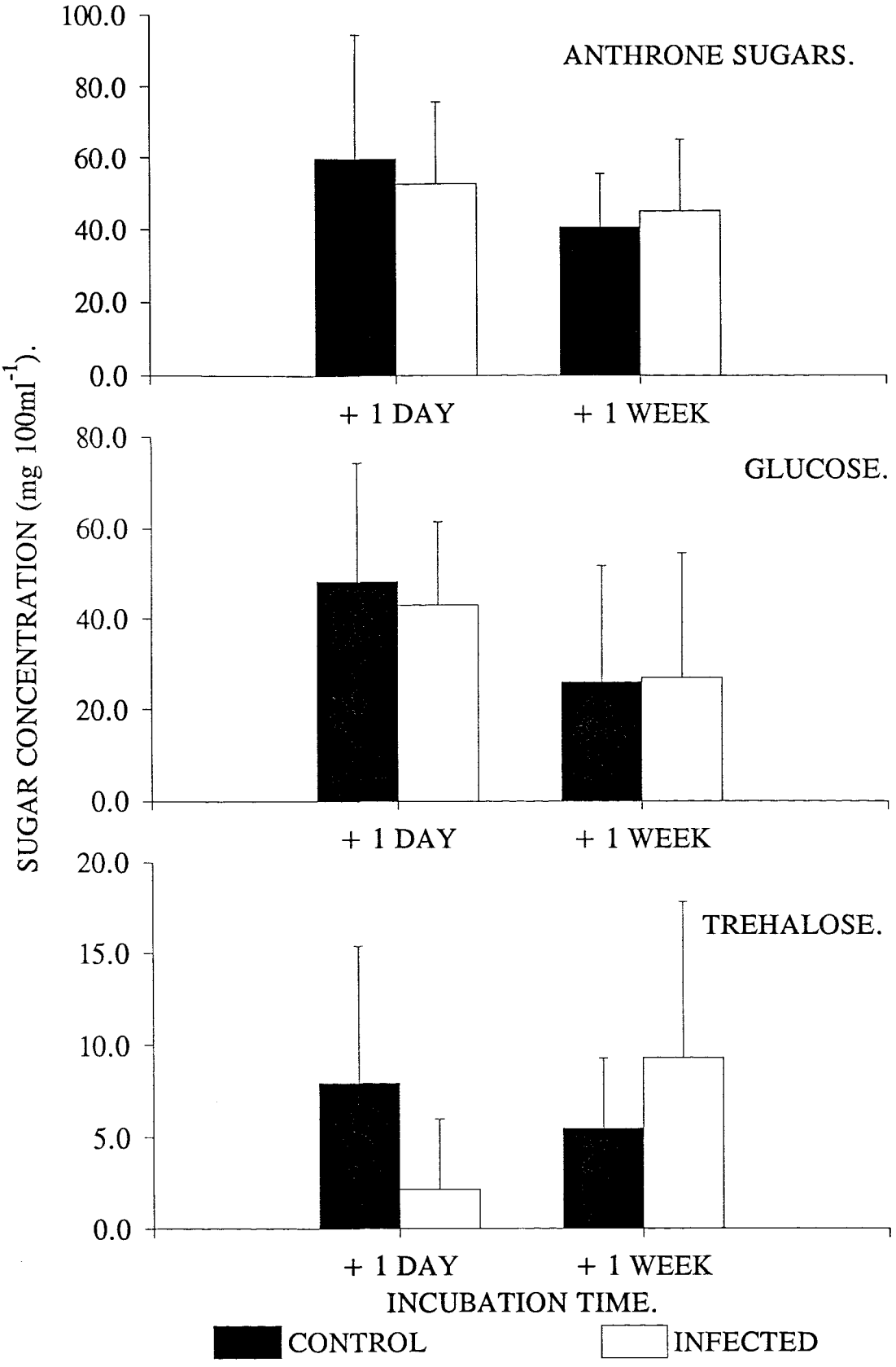

FIGURE 4.03 Breakdown of figure 4.02 showing changes in the different sugar groups measured and highlighting the large standard deviations of the data.

4.3.2 HAEMOLYMPH HYDROGEN PEROXIDE CONCENTRATIONS.

Fluctuations in the concentration of haemolymph hydrogen peroxide have been summarised in table 4.03 and figure 4.04 where it is seen that there was an increase in circulating hydrogen peroxide after a week incubation in the case of the infected crabs. The mean hydrogen peroxide concentration in the infected crabs after a week incubation (33.49 mg. per litre) was significantly higher than the control animals (16.22 mg. per litre, Mann-Whitney Rank Sum Test, $P < 0.05$). All other statistical comparisons proved non significant at the $P = 0.05$ level.

As has been previously hypothesised (see chapter 2, figure 2.09) the consumption of sugar in oxidative metabolism may be linked to the production of hydrogen peroxide as an end product. In this instance however, there is no correlation between the concentration of hydrogen peroxide and glucose or total sugar (Pearson Product Moment Correlations = 0.036 and 0.037 respectively, $P > 0.05$.)

INCUBATION PERIOD	CONTROL		INFECTED	
	[HAEMOLYMPH HYDROGEN PEROXIDE] (mg. l^{-1})	N	[HAEMOLYMPH HYDROGEN PEROXIDE] (mg. l^{-1})	N
+ 1 DAY	19.00 ± 10.78	10	22.34 ± 9.37	12
+ 1 WEEK	16.22 ± 7.26	6	33.49 ± 18.18	11

TABLE 4.03 Changes in haemolymph hydrogen peroxide following infection with the bacteria *Listonella anguillarum*. All values represent the mean ± one standard deviation of N observations.

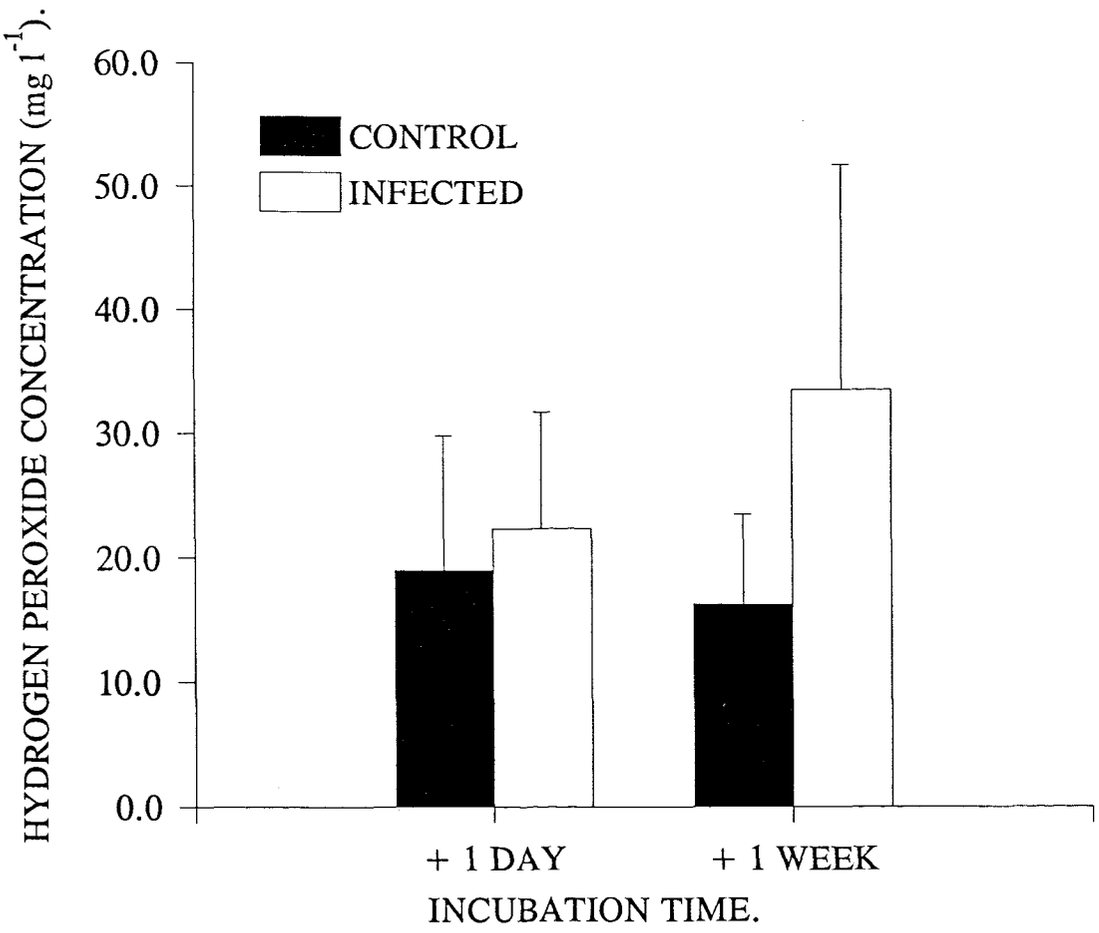

FIGURE 4.04 Variation in haemolymph hydrogen peroxide during a week incubation showing means ± one standard deviation of the collected data.

4.3.3 HAEMOCYTE POPULATIONS.

Changes in the circulating haemocyte population following infection are shown in figure 4.05 with the summarised data being presented in table 4.04. There was a significant decrease in the mean THC after a week incubation for the infected crabs. The THC of 20.76 x 10^6 cells per ml. in the infected crabs after a week was significantly less than the THC of infected crabs one day after infection (46.41 x 10^6 cells per ml.) and the THC of control crabs a week after inoculation (33.74 x 10^6 cells per ml.).

The changes in cell types which make up the total haemocyte population are shown in figure 4.06a which illustrates the ratio of granulocytes to hyalinocytes and also the changes in number of granulocytes and hyalinocytes (figure 4.06b & c). Statistical analysis revealed a significant increase in the ratio of granulocytes to hyalinocytes for the infected crabs after a week incubation (significantly higher than both the "control + 1 week" and "infected + 1 day" values; *t*-tests, $P < 0.05$).

To conclude, following bacterial infection there was a decrease in the circulating THC of infected crabs mostly due to a preferential removal of hyalinocytes from circulation leading to an increase in the ratio of granulocytes to hyalinocytes. In control organisms although there was a decrease in THC a week after sham injection the ratio of granulocytes to hyalinocytes remained approximately 2.5

4.3.4 PHENOLOXIDASE ENZYME ACTIVITY.

It was noted in chapter two that the enzyme phenoloxidase occurs as a zymogen within the granulocytes of decapods. To this end in order to measure any activity at all the HLS samples required treatment with trypsin elicitor (see section 2.2.2.4). The data presented in table 4.05 and figure 4.07 indicate the activity of the trypsin treated samples minus the activity of the cacodylate treated controls and demonstrate the extreme variability in phenoloxidase enzyme activity. Consequently none of the statistical comparisons proved significant at the $P <$

CONTROL, SHAM INJECTED CRABS.				
INCUBATION PERIOD	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
+ 1 DAY	35.70 ± 15.70	17.04 ± 7.86	52.06 ± 23.07	9
+ 1 WEEK	23.97 ± 8.34	9.78 ± 4.99	33.74 ± 13.22	7
BACTERIALLY INFECTED CRABS				
INCUBATION PERIOD	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
+ 1 DAY	33.50 ± 15.51	12.90 ± 5.90	46.41 ± 19.44	8
+ 1 WEEK	16.77 ± 4.30	3.99 ± 0.94	20.76 ± 5.14	6

TABLE 4.04 The affect of bacterial infection on the circulating haemocyte population during the week following a bacterial inoculation. Table shows the mean ± one standard deviation of N observations.

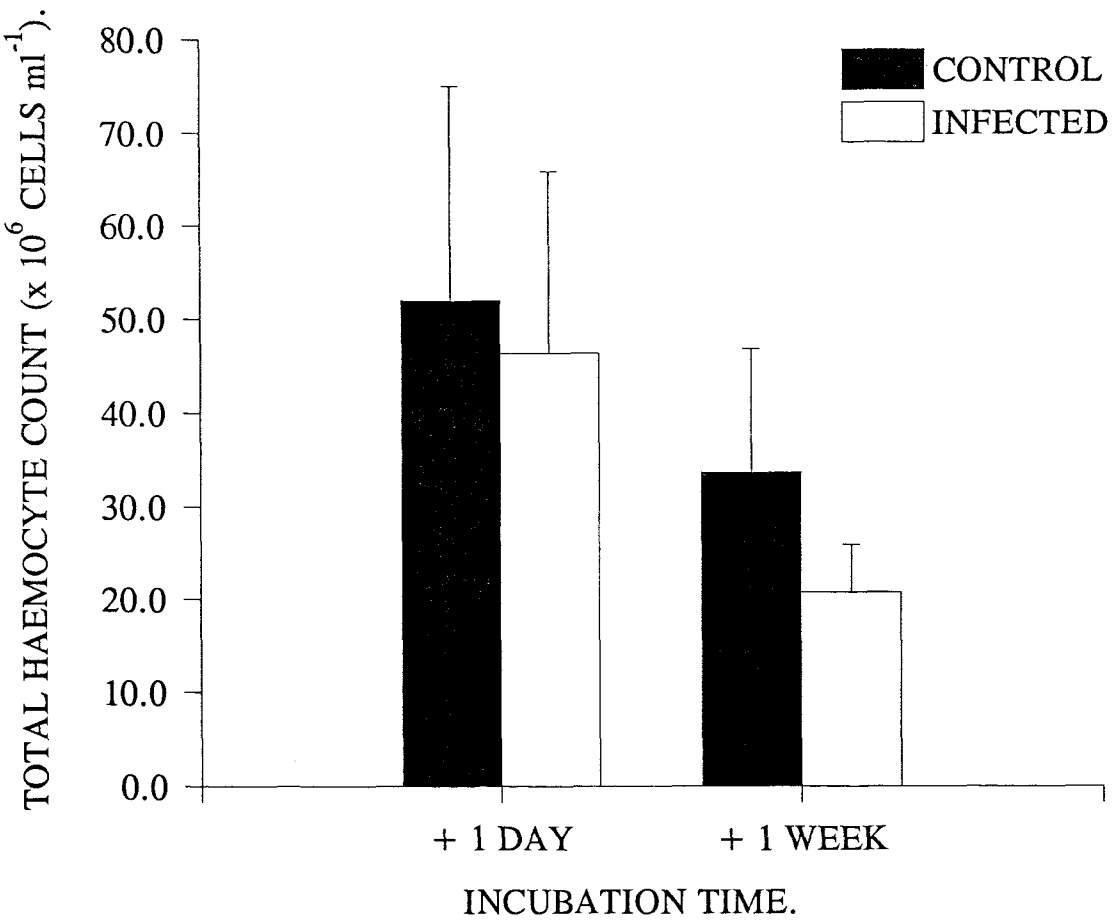

FIGURE 4.05 Changes in the circulating haemocyte population following infection. Data presented as shown in table 4.04.

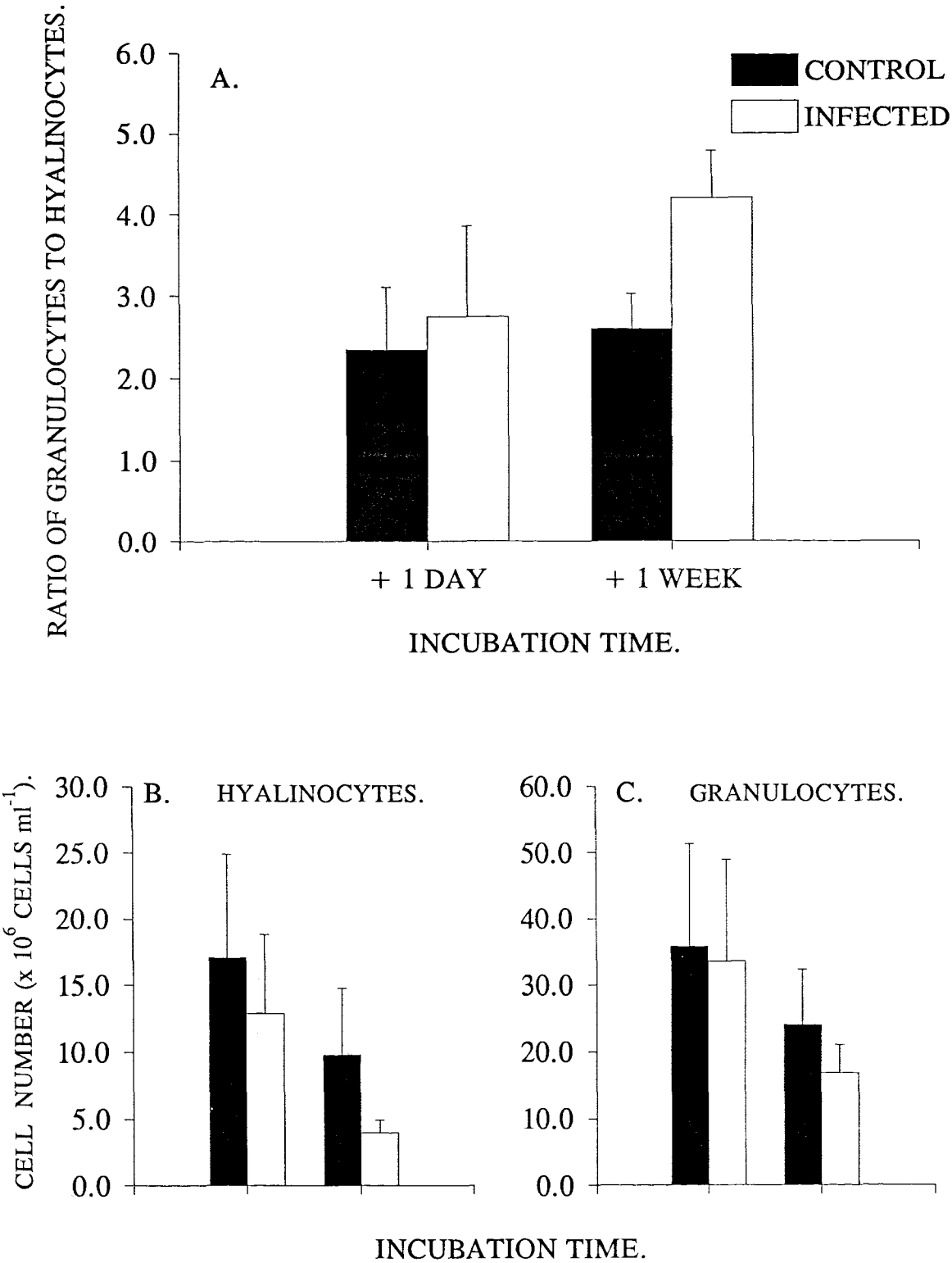

FIGURE 4.06 a. Variation in the composition of the circulating haemocyte population showing the ratio of granulocytes to hyalinocytes. b & c. The changes in actual cell numbers involved.

0.05% level. There was however, a clear depletion of the cellular store of zymogen a day after infecting crabs with the bacteria *L. anguillarum* (20.92 units per mg. protein in infected animals compared to 56.74 units per mg. protein in control animals). This reduction was then reversed after a week's incubation with the infected crabs having slightly more prophenoloxidase stored within the granulocytes (47.00 units per mg. protein) than the sham injected control crabs (28.54 units per mg. protein).

4.3.5 HISTOPATHOLOGY OF GILL TISSUES.

Comparative gill morphologies are shown in plates 4.01 and 4.02 from which it can be seen that there are clear differences in the gill lamellae structure between the control and infected crabs.

The gill lamellae of control crabs (plate 4.01) were typically composed of sinuses separated by pillar cell junctions (Smith & Ratcliffe, 1980a) with numerous free haemocytes visible. The lamellar structure of bacterially infected crabs, in contrast, shows some necrosis and degeneration of structure and a complete occlusion of the sinuses. Further to this the filaments show considerable inflammation in comparison to the control gills. Cellular clumping is evident in places as has been previously described (Smith & Ratcliffe, 1980a; see also plate 4.02) and a thicker layer (8-10μm.) of mucin (blue/purple coloured layer in both plates) on the surface of the gill lamellae of infected crabs can also be defined.

INCUBATION PERIOD	CONTROL		INFECTED	
	HLS ENZYME ACTIVITY (UNITS. mg^{-1} PROTEIN)	N	HLS ENZYME ACTIVITY (UNITS. mg^{-1} PROTEIN)	N
+ 1 DAY	56.74 ± 97.18	12	20.92 ± 34.35	11
+ 1 WEEK	28.54 ± 33.86	11	47.00 ± 57.16	8

TABLE 4.05 Changes in haemocyte lysate supernatant (HLS) phenoloxidase enzyme activity (trypsin activated samples minus control sample activity) following infection with the bacteria *Listonella anguillarum*. All values represent the mean ± one standard deviation of N observations.

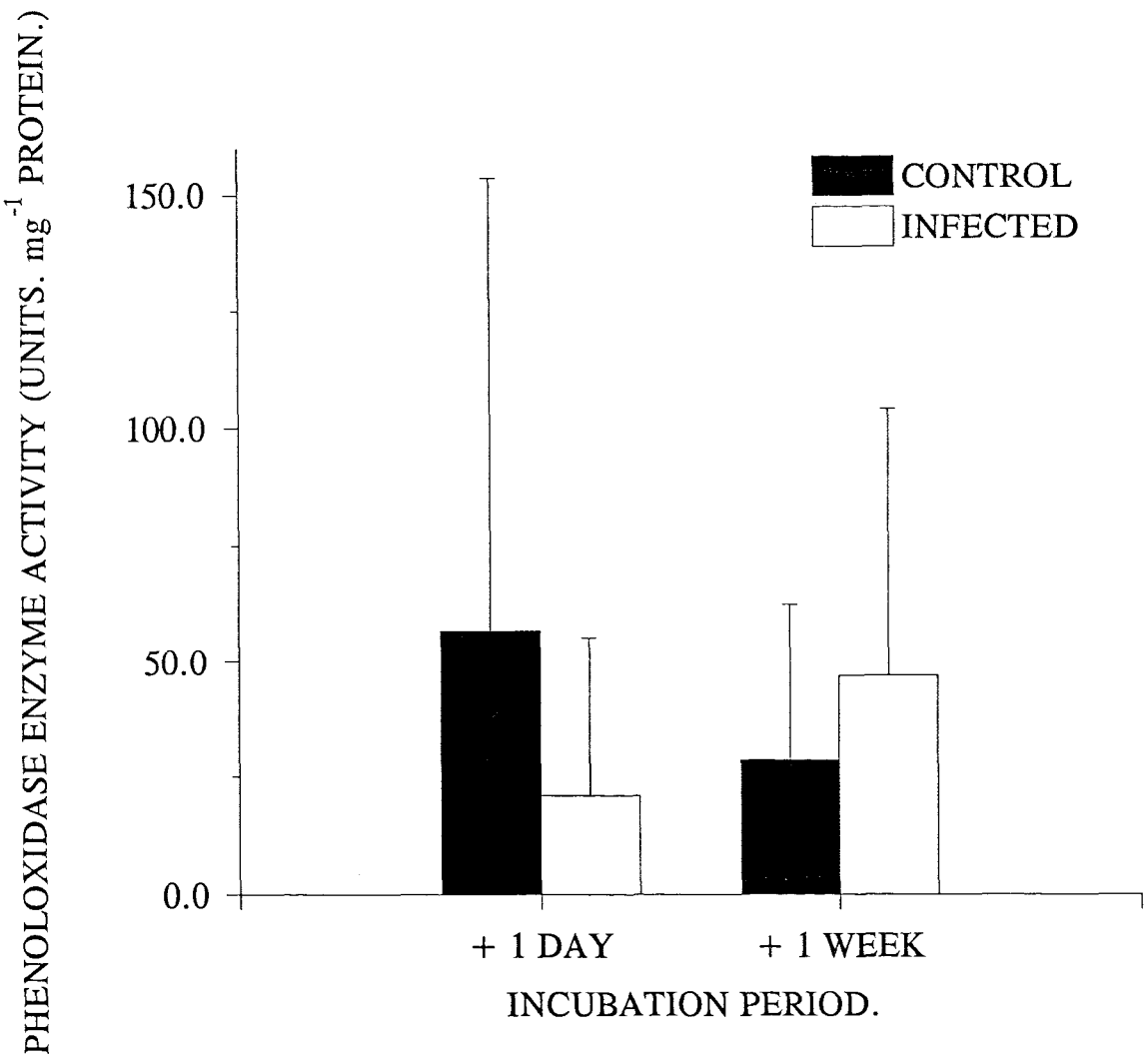

FIGURE 4.07 HLS phenoloxidase enzyme activities (trypsin activated samples-control sample activity) showing changes following bacterial infection over the week incubation.

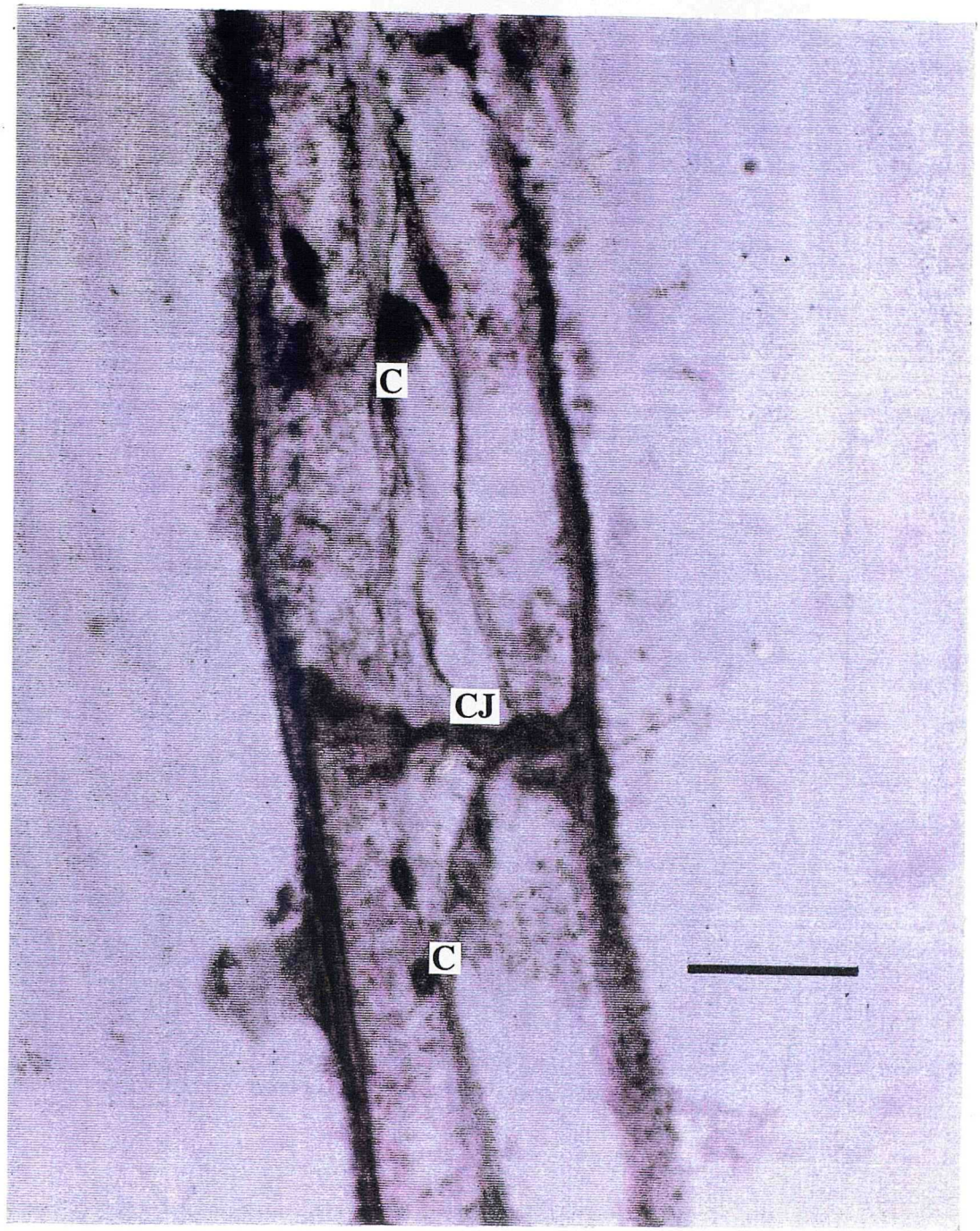

PLATE 4.01 Haematoxylin and eosin stained section of gill tissue from a control, *Carcinus* saline, injected crab (x 400). The intact structure of the gill filament and the unobstructed lumen are clearly evident. CJ = intact pillar cell junction, C = distributed haemocytes within the lumen of the filament. Scale bar = 20μm.

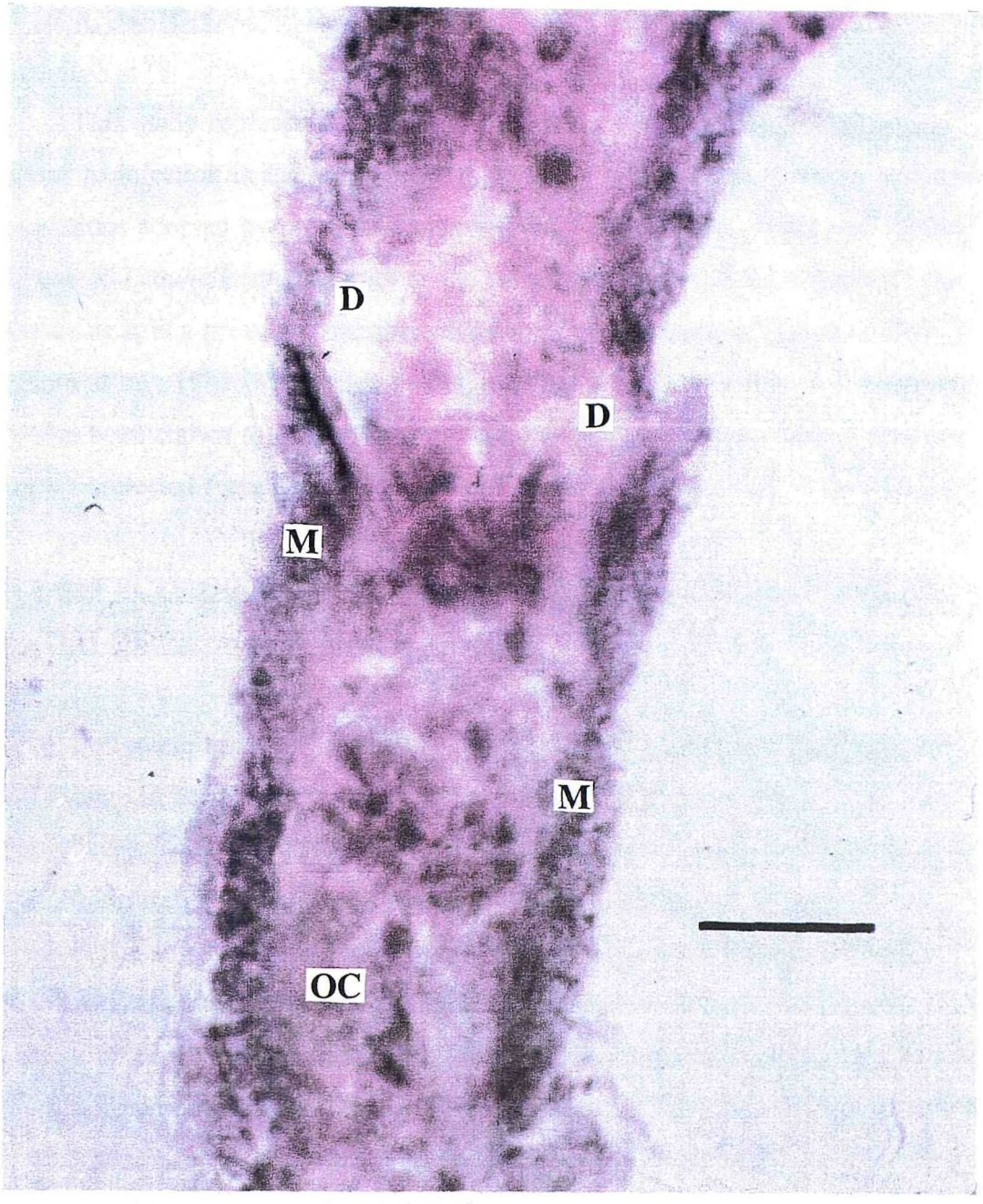

PLATE 4.02 Haematoxylin and eosin stained section of gill tissue from a *Listonella anguillarum* infected crab seven days after inoculation (x 400). Plate shows extensive degeneration and necrosis of the filament structure and a completely occluded lumen. All gills sectioned showed similar features. A thick (8-10μm.) layer of mucin is evident covering both external surfaces of the gill filament. M = layer of mucin, OC = occluded lumen, D = obvious areas of necrosis and degeneration of the tissue structure. Scale bar = 20μm.

4.4 DISCUSSION.

This study represents a novel investigation of the responses of *Carcinus maenas* to infection in that a live bacterium was used, as opposed to the denatured inoculations adopted by previous workers (Smith & Ratcliffe, 1980a and 1980b). The use of *Listonella anguillarum* as the infectious agent was appropriate in this instance as it is a prevalent member of the marine environment (Larsen, 1983; Ransom *et al.*, 1984; Muroga & de la Cruz, 1987; and Olafsen *et al.*, 1992) and has also been shown to occur naturally in both *Carcinus maenas* and in seawater samples collected from Southampton Water (see chapter 3).

4.4.1 EFFECTS OF PATHOGEN CHALLENGE ON THE METABOLIC STATUS OF *Carcinus maenas.*

Following bacterial infection it would initially appear that there was very little change in the carbohydrate status of the crabs (see figures 4.02 and 4.03). After a week incubation there was only a slight decrease in the total haemolymph sugar concentration of infected individuals which is mimicked by the control animals. The slight elevation a day after inoculation in both the infected and control animals may represent a general stress response to the injection and handling procedure (Telford, 1974b). However, the decrease in sugar over a week may also represent a utilisation of available circulating metabolite during a period of starvation. Kleinholz & Little (1949) have previously reported that there is no significant difference in the haemolymph sugar concentration in *C. maenas* starved for six days or twenty-four days, but they did not record any data for the first six days of starvation - the approximate duration of the incubation in the present study.

On closer inspection it can be seen however, that there were subtle changes in the composition of the circulating carbohydrate pool as shown in figures 4.02 and 4.03 and table 4.02. Firstly it is clear that the decrease in total sugar concentration was mainly due to a drop in the circulating glucose in both the control and infected individuals. It is also evident that the percentage contribution

of glucose to the total reducing sugars falls from approximately 80% a day after inoculation to approximately 60% a week after inoculation which, as suggested above, could either represent recovery after the initial shock response to the handling and injection or a utilisation of metabolic reserve during a period of starvation.

Trehalose sugars also underwent subtle changes during the week incubation. In the control crabs the percentage contribution of trehalose to the total sugar pool remained constant at approximately 13.5%, while in the infected crabs trehalose rose from approximately 4% to 20.72% of total sugar during the week. As discussed in the introductory chapter there are three different hypothetical roles for trehalose which may explain this initial depletion of the dimer and its subsequent recovery. It has been suggested (see review by Feolfilova, 1992) that trehalose may be involved in membrane stability and it is possible that the trehalose may have been involved in stabilising cell membranes immediately after infection or in the generation of new cells during the week following infection. In the first twenty-four hours following infection bacteria are sequestered in nodules within the gill filaments in *C. maenas* (discussed more completely below) - a process which involves cell destabilisation and lysis as well as aggregation and clotting. The idea that trehalose may be stabilising membranes during this time would therefore seem to be counter-intuitive. Also the potential utilisation of trehalose in the formation of new haemocytes over the week does not seem plausible as there was no apparent recovery in either granulocyte or hyalinocyte population during the week incubation (see below). In addition to this there was no obvious reduction in size of the cells counted (unpublished observations) indicating no apparent turnover or haemopoiesis.

A second possibility is that trehalose was acting as an intermediate store of carbohydrate between polysaccharides and glucose (Lillie & Pringle, 1980). Following infection the removal of trehalose may represent a complete conversion of this store to the more available glucose, a requirement which subsequently diminishes during the week leading to a recovery in trehalose concentrations after a week's incubation. It is however, difficult to verify this statement as the data really represent a snapshot of the changes rather than indicating the fluxes that

were taking place.

A final possibility is that the circulating trehalose acted as a quencher of roaming superoxide radicals produced as a non specific immune response to the bacteria (see Feofilova, 1992; for review). As can be seen from figure 4.04 in the infected crabs there was a significant increase in the levels of hydrogen peroxide a week after inoculating with bacteria. Bell & Smith (1993) have previously provided evidence for superoxide production in *C. maenas* hyaline cells assayed *in vitro* and more recently Song & Hseih (1994) demonstrated superoxide production following immunostimulation of tiger shrimp (*Penaeus monodon*) haemocytes. The end product of superoxide production is the formation of hydrogen peroxide which would explain the increase seen here. The increase in concentration of hydrogen peroxide does coincide with an increase in the concentration of circulating trehalose. Further evidence to support the idea of superoxide production can be found in the fact that in chapter two it was demonstrated that there was an inverse correlation between the utilisation of carbohydrate in routine metabolism and the generation of hydrogen peroxide as an end product (see section 2.3.1.2 and figure 2.09). However, as highlighted in the present results (see section 4.3.2) in this instance the two parameters are not related - the occurrence of hydrogen peroxide may be due to processes other than carbohydrate metabolism.

The concentration of other reducing sugars inferred from the difference between anthrone sugars and the sum of the contribution of trehalose and glucose tended to remain constant (approximately 7mg. per one hundred ml.) during the week incubation. The change in percentage contribution to the total circulating sugars merely reflects changes in the concentration of glucose and trehalose. The composition of this undefined group has been determined previously for *C. maenas* by Johnstone & Spencer-Davies (1972) using thin layer chromatography. These workers found that the other oligosaccharides in *C. maenas* were made up of the sugars fructose, fucose and maltose which was present as a consequence of the incomplete acid hydrolysis of glycogen during assay. Johnstone & Spencer-Davies (1972) separated the polysaccharide fraction by treating with concentrated ethanol prior to acid hydrolysis and from this work they demonstrated that the polysaccharide glycogen dominated the haemolymph carbohydrate pool constituting

80% of the total concentration. Glycogen breaks down to form glucose on hydrolysis and therefore it is important to note that a large proportion of the glucose measured in this study was due to hydrolysed polysaccharide.

4.4.2 EFFECTS OF PATHOGEN CHALLENGE ON IMMUNOCOMPETENCE.

As indicated in the results (see section 4.3.3) and mentioned briefly above, following a live bacterial infection there was a significant reduction in the number of circulating haemocytes (figure 4.05). Further to this it is evident from figure 4.06 that the decrease in THC was due to a preferential removal of the phagocytic hyalinocytes from circulation which, in turn, gave rise to a significantly higher granulocyte to hyalinocyte ratio in infected animals after a week's incubation.

Reductions in circulating haemocyte number have been demonstrated previously for decapod crustaceans following bacterial infections by a number of workers. Smith & Ratcliffe (1980a) reported a 90% decrease in circulating haemocyte number within the first hour of infecting *C. maenas* with the bacteria *Moraxella* spp. and *Bacillus cereus* and Martin *et al.* (1993) have recorded similar decreases (80%) in the penaeid shrimp *Sicyonia ingentis*. Both publications reported that this decline was transient and normal haemocyte numbers returned 72-96 hours. The continued decline in haemocyte number demonstrated in the current investigation and the absence of any haemopoeitic activity suggested above may be a function of the enhanced virulence of *L. anguillarum* used in this study. Indeed, Chisholm (1993) has found that haemocyte lysate supernatant (HLS) purified from *Carcinus* is not as effective at reducing the growth of *Listonella* cultures *in vitro* as it is for other species of bacteria such as *Psychrobacter immobilis*; further to this, in this study sub lethal doses of 10^5 bacteria per ml. were used which is four orders of magnitude lower than the doses of *Moraxella* spp. or *B. cereus* used by Smith & Ratcliffe (1980a).

The fate of the circulating haemocytes and injected particles once they are removed from the haemolymph has also been discussed by a number of different workers in the past, all of whom found that the major site of bacterial/haemocyte

accumulation was in the gill sinuses, although the hepatopancreas was also identified as a secondary site (Smith & Ratcliffe, 1980a & 1980b; White & Ratcliffe, 1980 & 1982; White *et al.*, 1985; and Martin *et al.*, 1993). Once sequestered within the gill sinuses, these bacteria/haemocyte clumps become melanised and necrotic and the processes of phagocytosis occur; although, even after a week cell clumps persist as has been demonstrated by Smith & Ratcliffe (1980a). The sequestration of bacteria within the gill tissue was confirmed in this present study by examining gill morphology a week after infecting with bacteria and it is evident from plates 4.01 and 4.02 that even after a week's incubation the lamellar sinuses remained occluded in the infected crabs. Plate 4.02 also indicates extensive degeneration of the gill tissue structure and an increase in the lamellar thickness which is suggestive of an inflammatory response to infection. White *et al.* (1985) reported that bacteria/haemocyte nodules may occupy forty percent of the lamellar sinuses which could lead to respiratory dysfunction and asphyxia in the short term. In the present study when an infection of 10^9 bacteria per ml. was used death occurred in all the infected crabs within five hours of inoculation, which would support this hypothesis. Another possible cause of haemolymph hypoxia could be the thickening of the mucus layer on the surface of the gill filaments demonstrated in plate 4.02. which may have served to increase the diffusion distance and impede effective gas exchange. This idea of respiratory dysfunction following inflammation and excessive mucus secretion remains a point of contention amongst researchers (Hutchinson, pers. comm.) and although it has been recently supported by Khan (1995) it really requires further experimental validation.

The localisation of the cell clumps within the gill tissue is considered to be a passive process associated with changes in the haemolymph flow across the lamellae (Smith & Ratcliffe, 1980a). The gill tissue is highly vascularised and foreign particles may be carried there with the haemolymph under positive pressure from the heart. Maynard (1960) has demonstrated a drop in the haemolymph pressure during flow through the gill lamellae and this may allow the haemocyte/bacteria aggregations to collect within the lacunae of the gill filaments. The initial formation of the cellular aggregations is considered to be a function of

the phenoloxidase enzyme system released from the refractile granulocytes on contact with bacterial pathogens (Smith & Ratcliffe, 1980a).

Following infection with bacteria a decrease in the activity of the circulating phenoloxidase enzyme was recorded in the short term (as shown in figure 4.07) followed by a recovery after a week's incubation. Although, as discussed in chapter two, the measurement of phenoloxidase activity does not directly refer to haemocyte number it is perhaps intuitive to expect that the enzyme would be similarly removed from the circulation and sequestered in the gill filaments where it would act to melanise the forming nodules and induce phagocytosis by the hyaline cells. Following a week incubation the level of circulating phenoloxidase recovers to above control values (47.00 units per mg. protein in infected crabs as opposed to 28.54 units per mg. protein in controls) even though the cell populations stage no such recovery. This would suggest that those granulocytes that do remain in circulation contain more inactive zymogen than at the start of the incubation and this may represent some form of non specific acquired immunity.

The concept of immunostimulation of crustaceans has been suggested before by Sung *et al.* (1994), who recently demonstrated that a temporary resistance to vibriosis may be induced in the tiger shrimp (*Penaeus monodon*) following inoculation with β-1,3 and β-1,6 glucans, molecules which occur in bacterial cell walls and which promote the activation of the zymogen prophenoloxidase (Söderhäll, 1982; and Johansson & Söderhäll, 1989). This present study is however, the first occasion in which the possibility of immunostimulation following non-lethal live infection has been suggested.

It can be concluded from the data presented in this chapter that following a live bacterial infection there was a short term reduction in the immunocompetence of *Carcinus maenas*, as indicated by a decrease in the number of circulating haemocytes and a depletion of prophenoloxidase reserves. In the medium term prophenoloxidase reserves recover, although without a concomitant recovery of the haemocyte population.

The data also indicate that following a sub-lethal infection of *Listonella anguillarum* there were few unequivocal changes in the carbohydrate status of *C. maenas* although subtle changes in sugar composition were apparent. These changes, as discussed, may reflect metabolic adaptation to starvation or a self protection mechanism against the activity of non specific immune defence processes such as superoxide production.

In the present investigation crabs were infected with *Listonella anguillarum* by injection via the fourth pereiopod in accordance with the method described by Smith & Ratcliffe (1980b). These workers also investigated the distribution of particles following injection at other sites around the body finding that inoculations through the cheliped produced the same gill sequestration as seen in the current work. Injections of carmine particles directly into the pericardial sinus through the carapace initially resulted in the dye collecting in the heart before it moved onto the gill filaments although this method of infection frequently caused the death of the experimental individual due to excessive haemolymph loss (Smith & Ratcliffe, 1980b). It would seem from these reports that in general the gills are the major sequestration site for xenobiotics in *Carcinus maenas* irrespective of the infection site. One other popular method of inoculation used by fish immunologists is that of "bath infection" (Tatner & Horne, 1983; Ransom *et al.*, 1984; Miyazaki, 1987; Muroga & de la Cruz, 1987; and Anderson & Siwicki, 1994). Whilst the ultimate origin of any infection in aquatic organisms tends to be from pathogens within the water column, for crustaceans infection only occurs once the carapace has been breached exposing the internal environment (reviewed by Sindermann & Lightner, 1988; and Stewart, 1993). As a consequence crabs were inoculated with bacteria by injection in the current study as this provided a more realistic and reliable means of producing a consistent infection.

Of course in the natural environment organisms not only face stresses imposed following pathogenic infection but are also faced with the problems of anthropogenic inputs such as heavy metal and hydrocarbon pollution. The following chapter considers the response of *C. maenas* to the stresses associated with exposure to the water soluble fraction (WSF) of crude oil.

CHAPTER FIVE

EFFECTS OF HYDROCARBON EXPOSURE ON THE CONDITION OF *Carcinus maenas*

CHAPTER FIVE

EFFECTS OF HYDROCARBON EXPOSURE ON THE CONDITION OF *Carcinus maenas*

5.1 INTRODUCTION.

Petroleum contamination of the marine environment routinely occurs from a variety of sources, of which atmospheric inputs form a significant proportion (Anderson *et al.*, 1974a). Chronic low level contamination of estuaries and coastal waters however, primarily originates from near-shore ship operations and via urban and industrial sewage. On a more intermittent basis, discharge of ballast water from tankers and accidental spills from offshore wells and shipping disasters, such as the "Torrey Canyon" in 1967 and the recent "Braer" incident in 1993, may seriously contaminate localised regions of the coastal zone.

An insight into the impact of hydrocarbon pollution on marine and estuarine species may be gained from consideration of the four types of biochemical and chemical investigation described by Anderson *et al.* (1974a):

> *1) Short term toxicity studies to determine the range of tolerance to the pollutant, to set exposure levels for sub lethal studies, and to evaluate the significance of environmental concentrations.*

This has been, and remains, the most common form of research into oil pollution and there are a plethora of reviews and papers that have been published on the subject (for example see: Lockwood 1976; Wolfe *et al.*, 1977; and Abel, 1991 for reviews). More specifically Anderson *et al.* (1974a) have reported the median lethal concentration (LC_{50}) for the sheepshead minnows (*Cyprindon variegatus*), the grass shrimp (*Palaemonetes pugio*), and the brown shrimp (*Penaeus aztecus*), exposed to a variety of water soluble fractions (WSF) from a range of crude oils, such as South Louisiana, Kuwait, No. 2 fuel oil, and

Venezuelan bunker C oil. Laughlin & Neff (1977) have demonstrated that the survival of larvae of the horseshoe crab (*Limulus polyphemus*) is reduced by exposure to increasing concentrations of the WSF fraction of No. 2 fuel oil. These workers also demonstrated that exposure to WSF resulted in protracted development times and a reduction in size of the surviving larvae. Similarly Caldwell *et al.* (1977) reported a decrease in the percentage survival of the zoeae of the Dungeness crab (*Cancer magister*) exposed to concentrations of greater than 1.1 milligrammes per litre of benzene and 0.0049 milligrammes per litre of crude oil (quoted as napthalene equivalents). Sharp *et al.* (1979) later investigated the hatching survival of the killifish (*Fundulus heteroclitus*) exposed to a 25% dilution of the WSF fraction of No. 2 fuel oil indicating that there was a reduction in the percentage success as the duration of exposure increased.

2) Organism - environment transfer studies to determine the rates of accumulation and release of a pollutant by an organism.

This type of research has also received considerable attention in the past. Fossato (1975) demonstrated the inability of mussels (*Mytilus galloprovincialis*) to completely eliminate hydrocarbons from their tissues on transfer to clean seawater. Even after extended incubations of up to fifty days a residual 12% of the initial body burden remained; further to this, Fossato (1975) demonstrated that the depuration process was temperature independent. Anderson *et al.* (1974a) comprehensively investigated uptake and release of oil in water dispersions of a variety of crude oils in the Pacific oyster (*Crassostrea virginica*) and the estuarine clam (*Rangia cuneata*), demonstrating that depuration of these animals, to undetectable levels of contamination, took between ten and fifty-two days. These workers were also able to show that the initial rate of hydrocarbon loss was a decreasing exponential function of time and that the napthalenes were the last compounds to be released. Anderson *et al.* (1980) have also modelled the uptake of crude oil components in the grass shrimp (*Hippolyte clarkii*), the coonstriped shrimp (*Pandalus danae*), and the mysid (*Neomysis awatschensis*) demonstrating that uptake asymptotically increased eventually reaching a plateau level determined

by the exposure concentration and the metabolic rate of conversion of the hydrocarbons *in vivo*.

The third recommended type of research suggested by Anderson *et al.* (1974a) was that based on the collection of data in the field:

> *3) Field studies - to compare the responses of marine animals to natural and perhaps chronic exposures to pollutants with responses observed in the laboratory, and to determine the effects of pollutants on the metabolism and structure of marine communities.*

Whilst this is the ideal endpoint of any biomonitoring study, laboratory studies do, after all, need to be extrapolated to the natural environment, realistically this means of experimentation is only practicable following natural disasters. Gilfillan (1980) made use of the minor "Tamano" oil spill in Casco Bay, Maine in 1972 to study hydrocarbon pollution in the mussel (*Mytilus edulis*) and the clam (*Mya arenaria*). George (1993) and Rusin *et al.* (1993) have similarly made use of the "Braer" oil spill in January 1993 to study the effects of hydrocarbon pollution in the field.

The fourth and final type of research suggested by Anderson *et al.* (1974a) related to the study of the physiological responses to oil pollution:

> *4) Physiological studies - to determine the extent and nature of modification of metabolic parameters in response to exposure to sublethal concentrations of the pollutant.*

At the time of these recommendations by Anderson *et al.* (1974a) the physiological responses of marine organisms to the stress induced by exposure to sublethal concentrations of hydrocarbons was poorly understood. Since 1974 however, this area of research has proved to be of key interest to marine physiologists.

Anderson *et al.* (1974a) followed up their recommendations by investigating the physiological responses to oil exposure and demonstrated a

reduction in the respiratory activity of *Palaeomnetes pugio* and *Penaeus aztecus*. These observations were later supported by O'Hara *et al.* (1982), who demonstrated reduced respiration in oil exposed *C. maenas* and Sabourin & Tullis (1982) who exposed the mussel (*Mytilus californicus*) to benzene, toluene, and benzo-α-pyrene (BaP). These workers showed that there were significant reductions in the heart rate at concentrations of 100ppm BaP, 50ppm benzene and 100ppm toluene and also reduced respiratory activity at 50ppm benzene, 1ppm BaP and 10ppm toluene. Conflicting results were however, reported by Laughlin & Neff (1977) who demonstrated an increase in the respiratory activity of the larvae of the fiddler crab (*Uca pugilator*) following exposure.

Other workers have investigated the effects of oil exposure on the moulting process in marine invertebrates. Mecklenberg *et al.* (1977) have demonstrated a complete cessation of moulting in both the coonstriped shrimp (*Pandalus danae*) and the king crab (*Paralithodes comtschatica*). O'Hara *et al.* (1985) later demonstrated that benzo-α-pyrene, a component of crude oils, inhibited cholesterol metabolism to steroids (mainly ecdysones) in the hepatopancreas of the shore crab suggesting that this explained the influences of hydrocarbons on moulting.

The effects of marine hydrocarbon pollution on scope for growth measurements have been investigated in the clam (*Mya arenaria*) and the mussel (*Mytilus edulis*) by Gilfillan (1980) who reported a significant decline in the scope for growth following the Tamano oil spill. Gilfillan (1980) did however, caution against the careless use and interpretation of scope for growth measurements caused by natural fluctuations associated with, for example, changes in temperature (Widdows & Bayne, 1971).

At a biochemical and molecular level of organisation enzyme induction following hydrocarbon exposure has been investigated. Kafafi *et al.* (1993) recently reported that enzyme induction followed binding of the hydrocarbons with a non-specific cytosolic protein known as the aryl hydrocarbon receptor (AhR), this bound receptor subsequently inducing the enzymes involved with the detoxification of hydrocarbons - the mixed function oxidases (MFOs). MFOs oxidise hydrocarbons to more polar substrates which are more excretable (Burns,

1976) and MFO induction has been demonstrated in zooplankton species by Walters *et al.* (1979) and in crabs by Lee *et al.* (1981). One MFO in particular has aroused much interest amongst researchers, the benzo-α-pyrene mono-oxygenase enzyme (BPM). BPM activity has been extensively investigated by O'Hara *et al.* (1982) who suggested that it might be adopted as a biomarker of hydrocarbon pollution. Williams & Hood (1988) have however recently questioned the usefulness of BPM as an indicator due to inconsistent variability in the baseline activity of the enzyme with respect to salinity cycles.

Most recently histological and *in vitro* techniques have been employed to investigate very subtle responses to hydrocarbon exposure. Zahn *et al.* (1995) and Kahn (1995) have both reported ultrastructural changes in the tissues of rainbow trout (*Onchorhynchus mykiss*) and the winter flounder (*Pleuronectes americanus*) respectively. Khan (1995), using an *in vivo* study, reported extensive hyperplasia and mucus secretion in gill tissues following hydrocarbon exposure and Zahn *et al.* (1995) used *in vitro* cell lines of rainbow trout hepatocytes to demonstrate time and dose dependent ultrastructural changes in the hepatocellular volume. These cellular modifications were apparent at lower exposure concentrations than those which produced significant reductions in neutral red retention time.

Interest has most recently focused on the effect of hydrocarbon and other pollution on the immunocompetence of marine invertebrates, particularly those of financial value (see review by Sindermann, 1993). The aim of the study summarised in this chapter was to fully investigate the consequences of exposure to hydrocarbon pollution on the metabolic status and immunocompetence of *C. maenas* and to assess the suitability of any of the measured indices as indicators of organic pollution. An innovation of the present study was the adaptation of the neutral red retention assay for use with sensitive crustacean haemocytes to investigate cellular stability and to possibly clarify the role of the haemolymph trehalose as discussed in chapter four.

The organic pollutant used in this study was the water soluble fraction of Wyche Farm crude oil. This oil is of local importance as it is pumped by pipeline from the Wyche Farm oilfield in Poole, Dorset to the British Petroleum refinery near Hamble on the shore of Southampton Water (see figure 1.02) from where it is exported by tanker (Parkhouse, pers. comm.).

5.2 METHODS.

5.2.1 COLLECTION, MAINTENANCE AND GENERAL EXPERIMENTAL PROCEDURE.

Crabs were collected from the salt marsh system around Hayling Island and maintained in aquarium holding tanks according to the protocol summarised in section 4.2.1. The acclimation and experimental conditions during this study were as follows: temperature 22.3 $\pm$ (SD) 1.5°C, salinity 33.8 $\pm$ 1.4‰ and the experiment was conducted over a three week period from 17th May to 10th June 1995, with one week dedicated for each of the haemocyte population studies and the metabolic assays. The phenoloxidase enzyme and neutral red retention assays were performed in the third week on separate batches of animals. For each week control and experimental groups of a minimum of six animals were incubated for seven days in communal aerated chambers. These glass-topped experimental chambers were not of a "flow through" design and, as a consequence, the water was changed every two days to prevent the excessive accumulation of excretory metabolites. In contrast to the previous study (chapter 4) the possibility of cross-contamination between infected crabs was not considered to be a problem and as a result separate chambers for each crab were not used. Crabs were transferred from the aquarium holding tanks to the experimental chambers twenty-four hours before any haemolymph was removed to allow the animals time to acclimate to the experimental environment.

Before any of the assays were carried out attempts were initially made to determine a lethal dose of the water soluble fraction (WSF) of Wyche Farm crude oil which was provided by SGS Redwood, Hamble. Seven groups (six crabs per group) were exposed to increasing concentrations of WSF (0% to 60% in 10% increments) for seven days and any mortalities were recorded. At a concentration of 60% WSF crabs began to die although even at this concentration 100% mortality did not occur. However, because of the constraints of time and the limited supply of Wyche Farm crude a solution of 50% WSF was used for the experimental incubations. At this concentration oil-induced mortality did not occur

but the crabs exhibited emersion responses similar to those described by Taylor & Butler (1973) for adult *Carcinus maenas,* held in shallow stagnant or polluted water. Further to this the incubation water became very dirty during the two days before it was changed, indicative of some sort of physiological stress.

5.2.2 PREPARATION OF WATER SOLUBLE FRACTIONS AND HYDROCARBON DETERMINATION.

Water soluble fractions (WSF) of Wyche Farm crude oil were prepared by adapting the methods of Anderson *et al.* (1974b) and Fisher & Foss (1993). One litre of crude oil was layered onto nine litres of aquarium seawater in a stoppered glass mixing vessel with a bottom-mounted tap. The mixture was stirred using a magnetic stirrer and bar, out of direct sunlight at room temperature, at a speed which maintained the mixing vortex to a depth of no more than six centimetres, which was approximately a quarter of the depth of the bottle. After stirring the mixture for twenty-four hours the solutions were left to separate for an hour before the water component was drawn off from the bottom via the tap. This water/oil mix was designated 100% WSF and was stored in foil-topped amber bottles at 5°C to prevent photo-oxidation and the loss of volatile fractions. Dilutions of 100% WSF were made using clean aquarium seawater for the incubations (see above).

Total hydrocarbon concentration in 100% WSF was determined using gas chromatography. Hydrocarbons were extracted from a sample of WSF that had been stored at 5°C for a period longer than a week (ie. not freshly made) using the method described by Law *et al.* (1988). Briefly, two litres of 100% WSF were mixed, by vigorous shaking for five minutes, with fifty millilitres of hexane. This was allowed to settle in a separating funnel before the bottom phase (mostly water) was returned to the storage bottle. The procedure was repeated with a second fifty millilitre aliquot of hexane and the two hexane/hydrocarbon samples were combined. The sample was analysed by Exxon Chemicals of Fawley, where it was reduced under nitrogen to form a residue and analysed using a high pressure liquid chromatography (HPLC) column.

5.2.3 METABOLIC AND IMMUNOLOGICAL INDICES OF ORGANISM CONDITION.

All of the measured indices were assayed using the protocols described in the previous chapter (section 4.2.2). During this study attempts were made to develop an assay to measure phenoloxidase enzyme activity in the plasma, all methods however, proved unsuccessful. This is discussed more completely in the following chapter.

5.2.3.1 NEUTRAL RED RETENTION ASSAY.

In vitro cell stability was determined using a modification of the neutral red (NR) uptake assay (Borenfreund & Puerner, 1984), as described by Reader *et al.* (1989). The modified assay measures the rate of loss of the supravital stain neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) from cells incubated *in vitro* after a period of dye loading.

Neutral red uptake.

Before the affects of imposed stressors on the retention of neutral red (NR) by cells could be investigated the optimum time period for dye loading had to be determined (Schild *et al.*, 1993).

To ensure that the harvested haemocytes remained viable throughout the incubation the samples of haemolymph were collected in citrate EDTA anticoagulant (Söderhäll & Smith, 1983). The dye loading procedure has been summarised in figure 5.01. Neutral red working solutions were made freshly immediately before use from a stock solution stored at 5°C (see appendix A.5). Briefly, 2ml. of neutral red solution was added to the haemolymph/anticoagulant mixture and incubated for approximately six hours at room temperature. At regular intervals during this incubation 600μl. aliquots were removed and spun at 450*g* for ten minutes. The supernatants were removed, 1ml. of destain solution (see appendix A.5) was added and the cell pellets were physically disrupted and

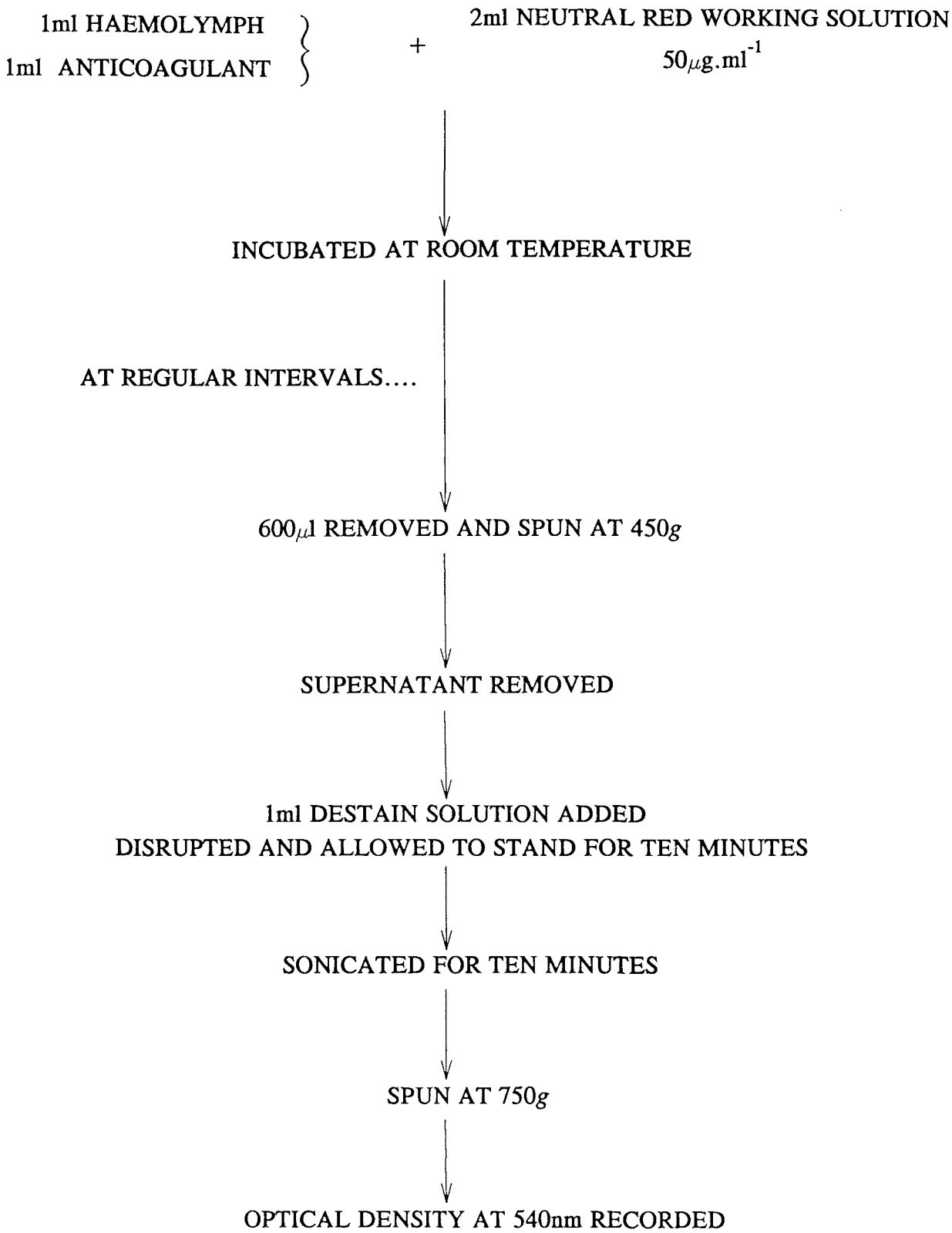

FIGURE 5.01 Experimental protocol adopted to measure the uptake of the dye neutral red by haemocytes of *Carcinus maenas*. Discussed more completely in the text.

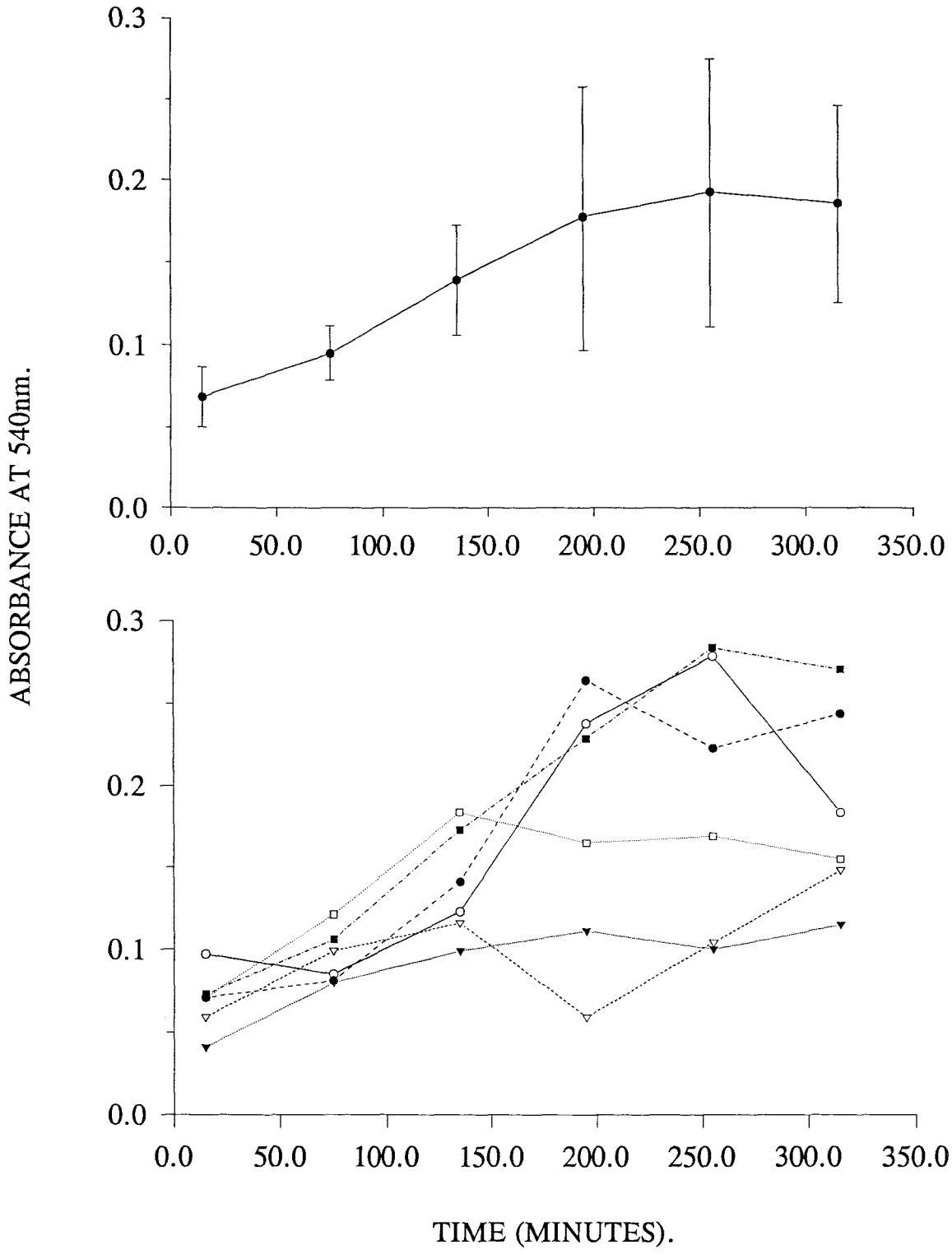

FIGURE 5.02 Uptake of the stain neutral red by *Carcinus* haemocytes represented as a function of time. Figure shows the combined data of six different samples which have also been plotted individually for completeness.

allowed to stand at room temperature for ten minutes. The assays were subjected to a further ten minutes of sonication before being centrifuged at 750*g*. The optical density of the remaining supernatants were measured at a wavelength of 540nm using the destain solvent as a blank.

Figure 5.02 summarises the results obtained from the dye loading study carried out on six separate haemolymph samples from which it can be seen that there was an increase in the optical density of the supernatants reaching a maximum after approximately three hours. As noted by Schild *et al.* (1993) for *Enteromorpha intestinalis* the degree of between sample variation in neutral red uptake increased after three hours, as evidenced by the larger standard deviations. Further to this statistical analysis of the present data (Kruskal-Wallis one way ANOVA on the non parametric data and subsequent Student Newman Keuls comparisons) showed there to be no significant change in the optical density after 135 minutes loading. In light of these two factors an optimal dye loading period of 2.5 hours was adopted.

Neutral red release.

After determination of the optimal dye loading period the rate of NR loss was determined for six crabs held in the aquarium as part of the preliminary study. As described above the haemolymph was collected in anticoagulant and incubated for 2.5 hours at room temperature with 2ml. of neutral red working solution. After 2.5 hours the samples were centrifuged at 180*g* for ten minutes and the supernatant (unabsorbed neutral red) removed. The loaded haemocytes were then suspended in 3ml. of *Carcinus* saline (Smith & Ratcliffe, 1978). At regular intervals 600μl. aliquots were removed and centrifuged at 450*g*, the supernatant was removed from each sample and the pelleted haemocytes which contained unreleased dye were destained as described in figure 5.03.

This preliminary study of the rate of NR loss from haemocytes showed it to be non linear (see figure 5.04). Two separate transforms ($\log_{10}$ and $\log_e$) of the data were attempted to determine which modelled the loss of dye more accurately

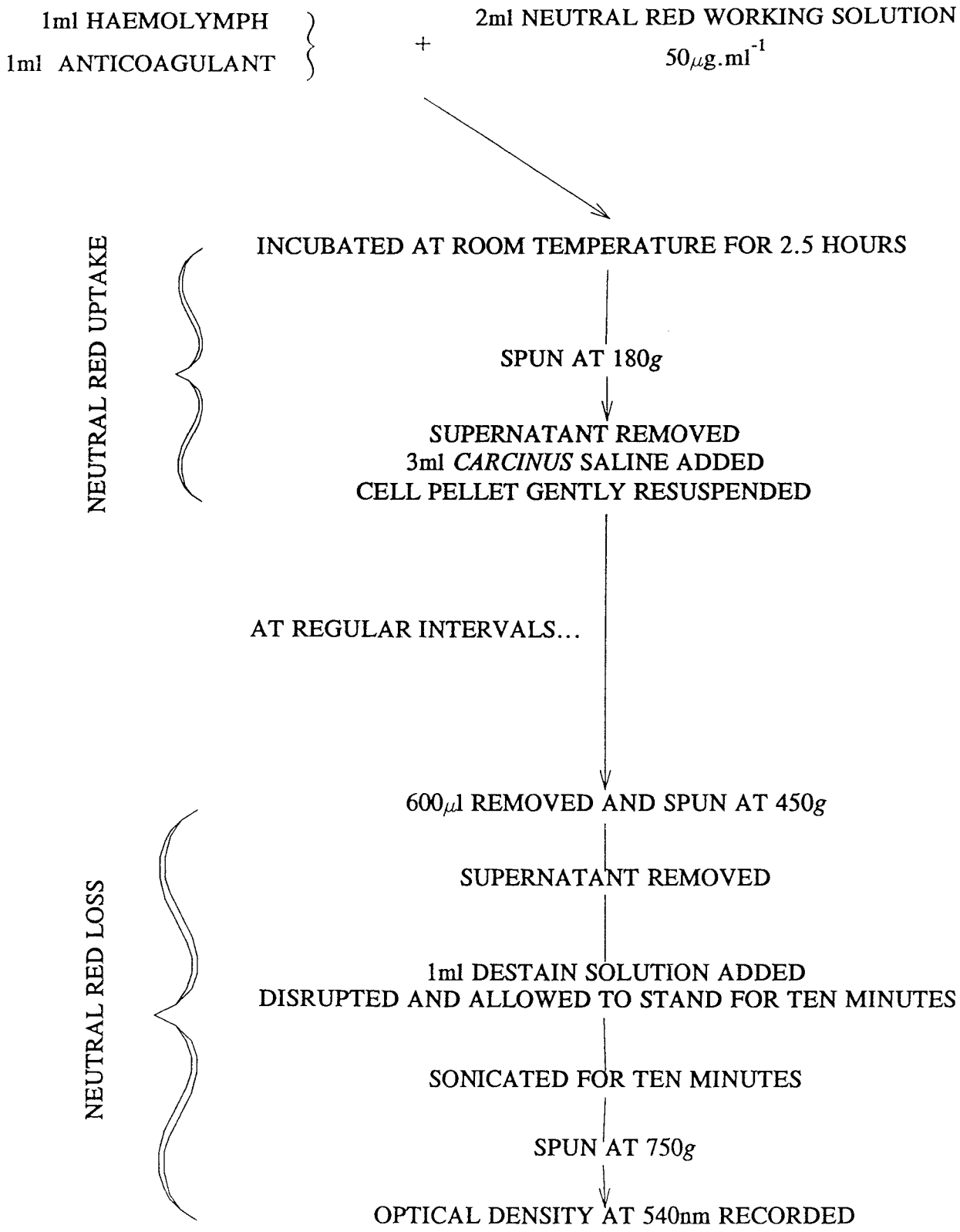

FIGURE 5.03 Experimental protocol adopted to measure the rate of neutral red release from loaded cells. The assay has two distinct parts, a "loading" phase and the "loss" phase. The protocol indicated represents the final methodology adopted for the experimental incubations.

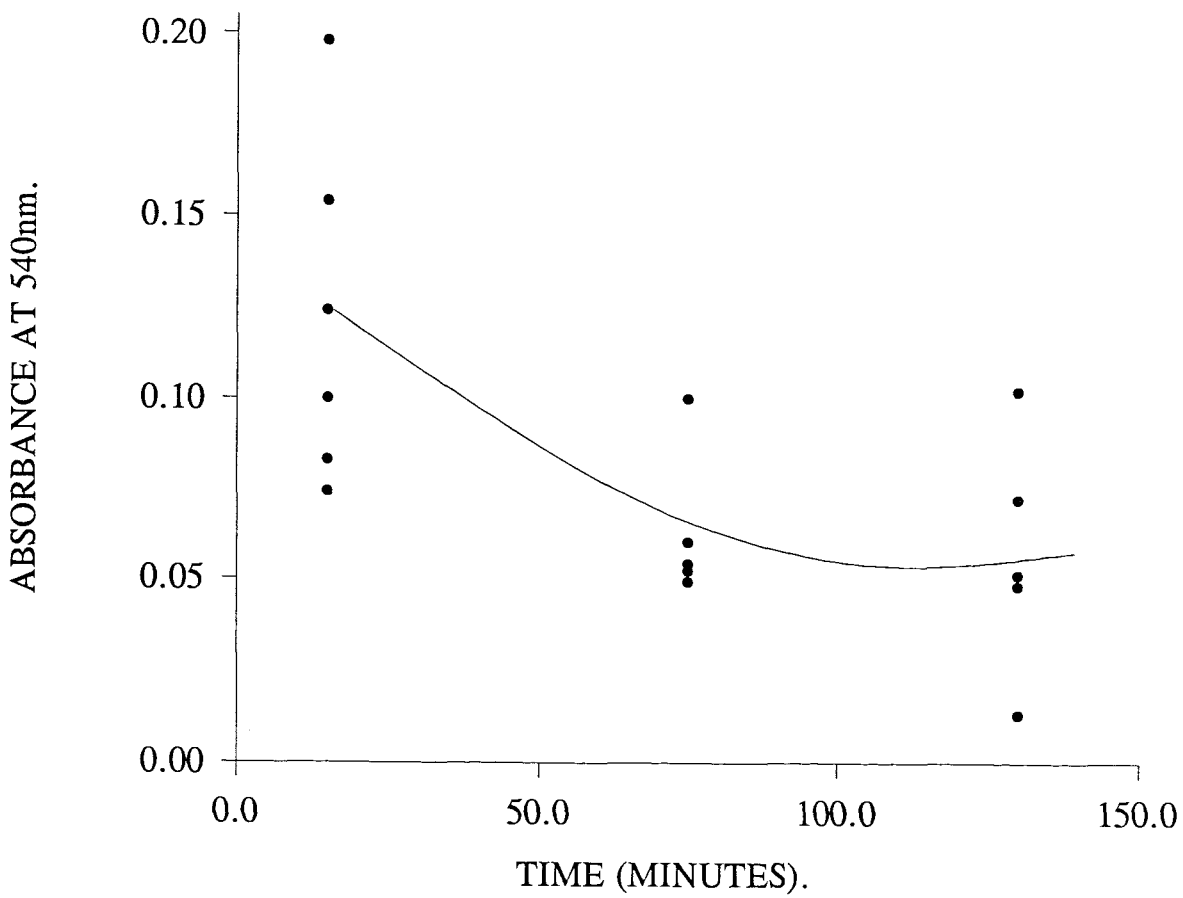

FIGURE 5.04 Data collected from the preliminary study on the rate of neutral red release as a function of time, showing the non linear release rate for the six individual haemolymph samples assayed.

(see figures 5.05 and 5.06).

Kvålseth (1985) and Scott & Wild (1991) have previously warned of the dangers of comparing the 'fit' of different transformed models using the coefficient of determination R^2 and have advocated the use of the following relation to produce directly comparable R^2 values:-

$$R^2 = 1 - (\Sigma(u - \gamma)^2/\Sigma(u - \bar{u})^2) \qquad (6)$$

u = measured dependent variable

$\bar{u}$ = mean of measured dependent variables

γ = value of dependent variables determined from regression relation.

Using this relation produced coefficients of determination of 0.355 ($\log_{10}$) and 0.511 ($\log_e$) from which it was concluded that the decay of neutral red from the haemocytes was best described as an exponential function of time. As a consequence all collected data from the hydrocarbon exposure incubations were transformed ($\log_e$) before comparison.

5.2.4 STATISTICAL ANALYSIS OF DATA.

The data collected in this study were analysed using the methods discussed in chapter 4 (section 4.2.4). Significant differences were once again observed when $P < 0.05$.

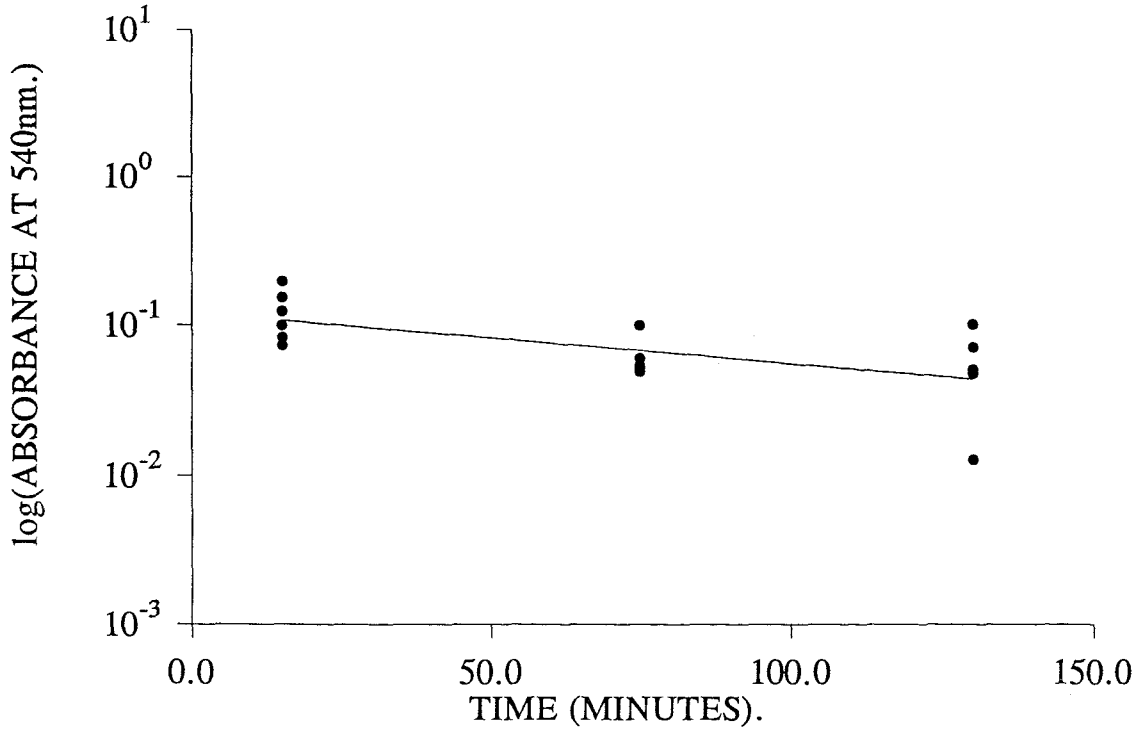

FIGURE 5.05 Logarithmic ($\log_{10}$) transformation of the absorbances of the six haemolymph samples presented in figure 5.04 showing the calculated regression line of the logged data.

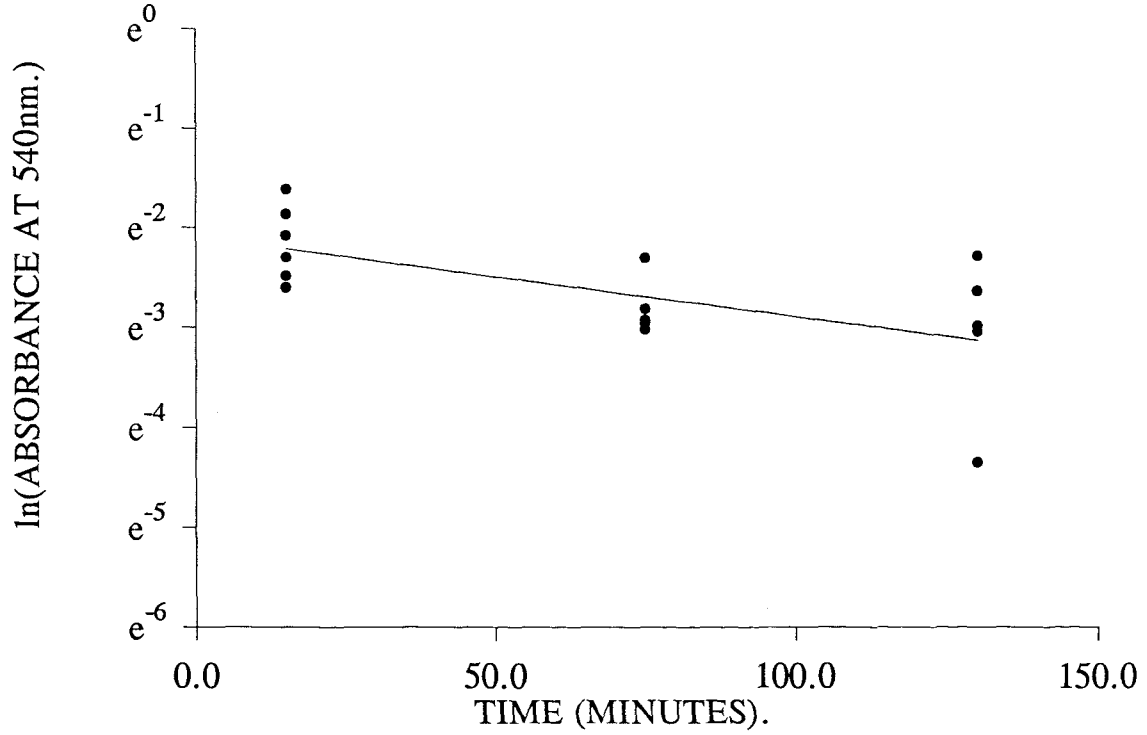

FIGURE 5.06 Logarithmic ($\log_e$ or ln) transformation of the data presented in figure 5.04 again showing the calculated regression line.

5.3 RESULTS.

5.3.1 SUMMARY OF THE HYDROCARBON ANALYSIS.

The elution profile from the HPLC indicated that the WSF was dominated by volatile low molecular weight hydrocarbons of eleven carbon atoms or less with a smaller contribution being made by hydrocarbons of seventeen to twenty-five carbon atoms which were probably lipids of biogenic origin. Trace amounts of high molecular weight organics (thirty-six to forty-five carbon atoms) were also seen on the elution profile and these probably derived from marine engine oils present in the aquarium seawater or from small droplets of crude oil that were present in the WSF either in suspension of attached to surfaces of the storage bottle. The total hydrocarbon concentration of 100% WSF was found to be 150ppb (Bianchi, pers. comm.).

5.3.2 INDICES OF METABOLIC STATUS.

Changes in the concentration of haemolymph sugars measured in this study have been summarised in table 5.01. The concentration of "other sugars" included in the table was inferred from the difference between the total sugar and the combined glucose and trehalose data. In two instances however ("control + 1 day" and "polluted + 1 day"), the combined glucose and trehalose values exceeded the total sugar concentration, which was anomalous. In these two cases the percentage contribution of glucose and trehalose were calculated using the glucose and trehalose data alone (i.e. quoting the contribution of glucose as a percentage of the combined glucose and trehalose concentration and not as a percentage of the total sugar concentration). These anomalies were probably caused by subtle differences in the different assay techniques and may also indicate operator error; for example: had the conditions for the trehalose assay not been optimal it may have caused incomplete hydrolysis of the sugars causing contamination of the trehalose with other oligosaccharides. This problem highlights the difficulty in comparing data from different assays conducted by different workers on separate occasions.

+ 1 DAY.						
SUGAR	CONTROL			POLLUTED		
	mg. $100ml^{-1}$	%	N	mg. $100ml^{-1}$	%	N
TOTAL SUGAR	12.92 ± 8.82	100.00	12	17.59 ± 7.80	100.00	12
GLUCOSE	7.11 ± 4.50	54.82	6	16.18 ± 6.33	70.66	6
TREHALOSE	5.86 ± 4.19	45.18	12	6.72 ± 3.41	29.34	12
OTHER SUGARS	**0.00**	**0.00**	-	**0.00**	**0.00**	-
+ 1 WEEK.						
SUGAR	CONTROL			POLLUTED		
	mg. $100ml^{-1}$	%	N	mg. $100ml^{-1}$	%	N
TOTAL SUGAR	29.49 ± 12.98	100.00	12	29.44 ± 9.14	100.00	12
GLUCOSE	10.56 ± 3.90	35.81	6	19.44 ± 7.43	66.03	6
TREHALOSE	9.26 ± 3.37	31.40	12	8.53 ± 4.70	29.97	12
OTHER SUGARS	**9.67**	**32.79**	-	**1.47**	**4.00**	-

TABLE 5.01 Changes in the haemolymph sugar composition of *Carcinus maenas* during a week incubation in 50% WSF. Table shows means ± one standard deviation of N observations. Figures in bold have been inferred as discussed in the text.

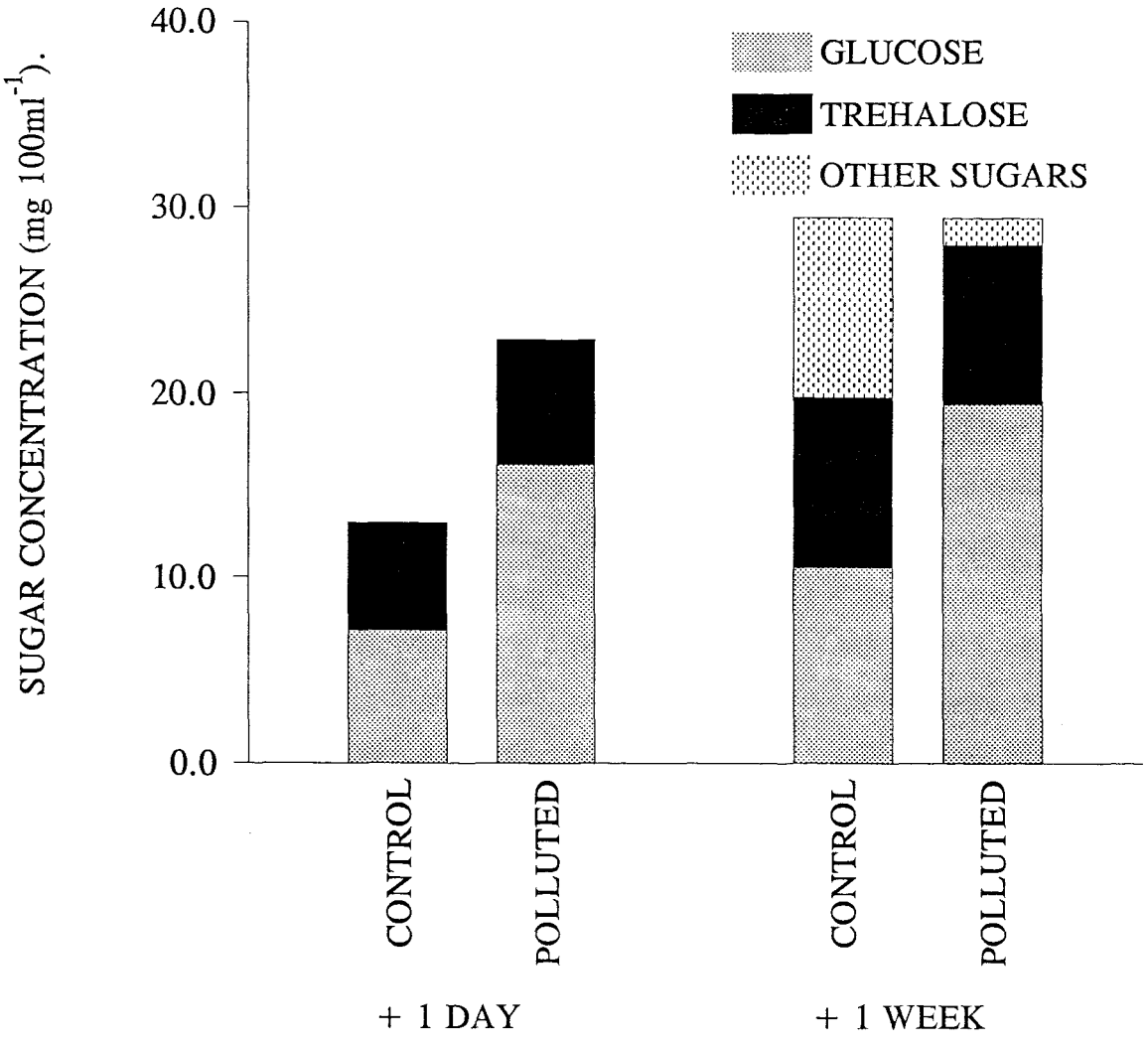

FIGURE 5.07 Haemolymph sugar responses to hydrocarbon exposure as summarised in table 5.01. Figure shows the mean sugar concentration, the standard deviations and sample sizes are summarised in table 5.01.

The data presented in table 5.01 have been summarised in figure 5.07 from which it can be seen that there were significant increases ($P < 0.05$) in the total haemolymph sugar in the control crabs during the week incubation from a mean of 12.92 $\pm$ 8.82 mg. per one hundred ml. to 29.49 $\pm$ 12.98 mg. per one hundred ml. (an increase of 128.25%), as well as in those crabs exposed to hydrocarbon pollution (from 17.59 $\pm$ 7.80 a day after initial exposure to 29.44 $\pm$ 9.14 mg. per one hundred ml. after seven days exposure, representing an increase of 67.37%).

It can be seen from table 5.01 that the increase in total haemolymph sugar was, in part, due to small increases in the circulating glucose concentration, evident in both the control and hydrocarbon-exposed crabs. In spite of this increase however, the percentage contribution of glucose to the total sugar pool decreased in both cases due to an increase in the "other sugar" component. Statistical analysis of the glucose data showed there to be a significantly higher glucose concentration in the hydrocarbon exposed crabs (16.18 $\pm$ 6.33 mg. per one hundred ml.) than the control organisms (7.11 $\pm$ 4.50 mg. per one hundred ml.) twenty-four hours after the initial exposure which was maintained throughout the seven day incubation period ("control + 1 week": 10.56 $\pm$ 3.90 mg. per one hundred ml.; "polluted + 1 week": 19.44 $\pm$ 7.43 mg. per one hundred ml., $P < 0.05$ with ten degrees of freedom).

For the trehalose sugars there was very little difference between the start and end of the incubations or between the control and hydrocarbon exposed animals. The only change which proved to be significant ($P < 0.05$) was the small increase in trehalose concentration in the control crabs during the week incubation. Table 5.01 also indicates very little change in the percentage contribution of trehalose made to the total circulating sugar pool. A decrease from 45.18% to 31.40% was seen over the week incubation for the control crabs, mostly due to an increase in "other sugars", and very little change was observed in the case of the hydrocarbon exposed crabs.

Fluctuations in the concentration of haemolymph hydrogen peroxide associated with an exposure to hydrocarbon pollution are indicated in table 5.02 and figure 5.08. Although, as illustrated in figure 5.08, changes were apparent as a consequence of a week's incubation in the experimental chambers there appeared

INCUBATION PERIOD	CONTROL		POLLUTED	
	[HAEMOLYMPH HYDROGEN PEROXIDE] (mg. l^{-1})	N	[HAEMOLYMPH HYDROGEN PEROXIDE] (mg. l^{-1})	N
+ 1 DAY	12.44 ± 6.40	12	12.15 ± 7.81	12
+ 1 WEEK	8.74 ± 5.06	10	6.89 ± 3.73	12

TABLE 5.02 Summarising changes in haemolymph hydrogen peroxide as a consequence of exposure to 50% WSF of Wyche Farm crude oil. All values represent the mean ± one standard deviation of N observations.

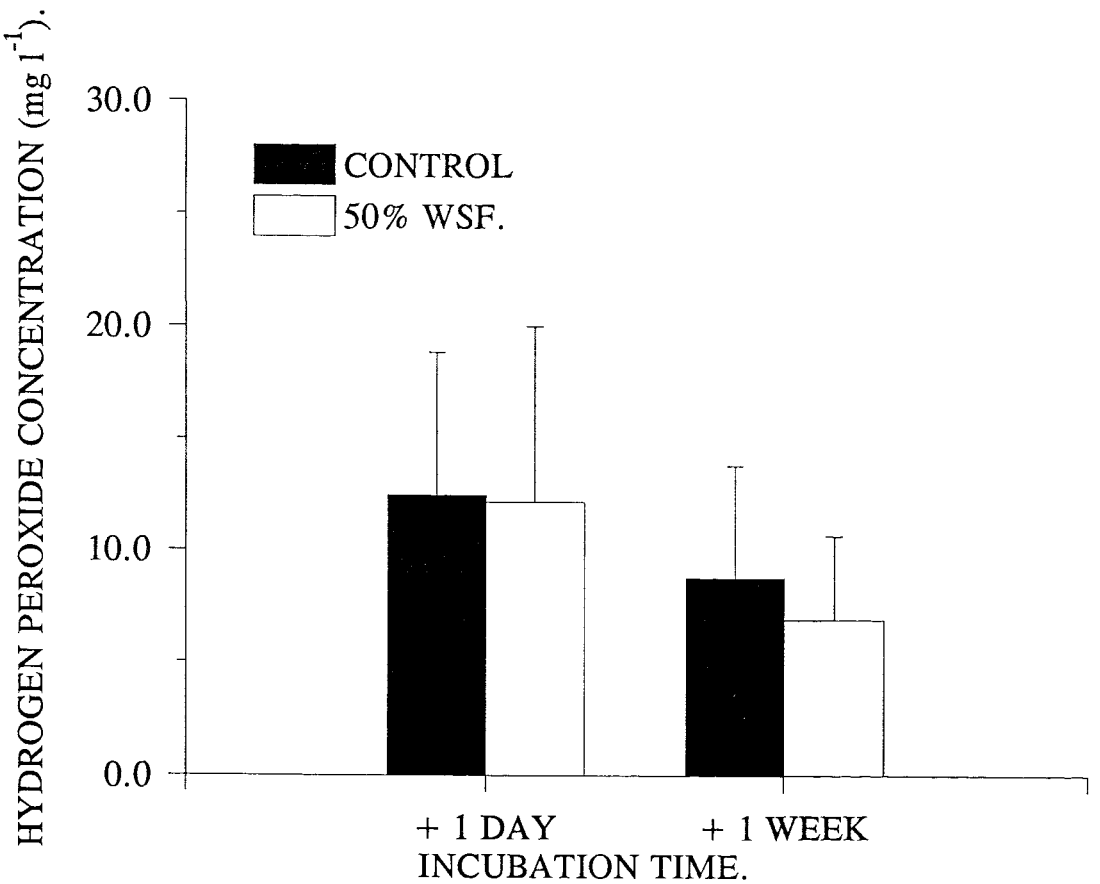

FIGURE 5.08 Haemolymph hydrogen peroxide changes following a week incubation with 50% WSF, showing the mean ± one standard deviation of the collected data. Sample sizes were variable and have been indicated in table 5.02

to be no obvious influence of exposure to hydrocarbons. Figure 5.08 indicates that for both experimental conditions there was a decline in the circulating hydrogen peroxide from approximately 12.3 mg. per litre on day one to approximately 7 mg. per litre on day seven. Statistical analysis revealed this reduction to be significant in the case of the hydrocarbon exposed crabs.

Pearson Product Moment correlations were performed on all of the metabolic data collected in this study. In this instance the only correlation that proved significant was between the concentration of haemolymph trehalose and the total sugar concentration (correlation coefficient = 0.489, $P < 0.001$, for forty-six observations.)

5.3.3 INDICES OF IMMUNOCOMPETENCE.

The effects of one week's exposure to 50% WSF on the composition of the circulating haemocyte population in *Carcinus maenas* have been indicated in table 5.03 and figure 5.09. Included in table 5.03 are data relating to the change in granulocyte and hyalinocyte cell populations from which the more convenient granulocyte: hyalinocyte ratio has been calculated, see figure 5.10.

From figure 5.09 it is evident that there was a reduction in the number of circulating haemocytes on exposure to 50% WSF ("control + 1 day": 84.29 $\pm$ 48.37 x 10^6 cells per ml.; "polluted + 1 day": 42.88 $\pm$ 19.96 x 10^6 cells per ml., $P < 0.05$) which was maintained throughout the week incubation in spite of a significant decrease in the haemocyte population in the control crabs ("control + 1 week": 53.58 $\pm$ 15.21 x 10^6 cells per ml.; "polluted + 1 week": 25.95 $\pm$ 9.13 x 10^6 cells per ml., $P < 0.05$). Figure 5.10 indicates that at the start of the incubation there were a higher proportion of hyalinocytes in circulation than at the end of the week in both the control and experimental groups evidenced by an increase in the granulocyte: hyalinocyte ratio from approximately 2.4 to approximately 4.2 during the week incubation.

Changes in the activity of the phenoloxidase enzyme following one week's exposure to 50% WSF of Wyche Farm crude oil have been indicated in table 5.04

CONTROL CRABS.				
INCUBATION PERIOD	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
+ 1 DAY	60.49 ± 36.61	23.79 ± 12.20	84.29 ± 48.37	6
+ 1 WEEK	41.49 ± 13.89	12.08 ± 5.68	53.58 ± 15.21	6
OIL EXPOSED CRABS.				
INCUBATION PERIOD	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
+ 1 DAY	29.57 ± 13.07	13.31 ± 7.42	42.88 ± 19.96	6
+ 1 WEEK	20.30 ± 9.02	5.65 ± 2.17	25.95 ± 9.13	6

TABLE 5.03 Summarising the affect of exposure to hydrocarbons on the circulating haemocyte population during the week incubation. Table shows the mean ± one standard deviation of N observations.

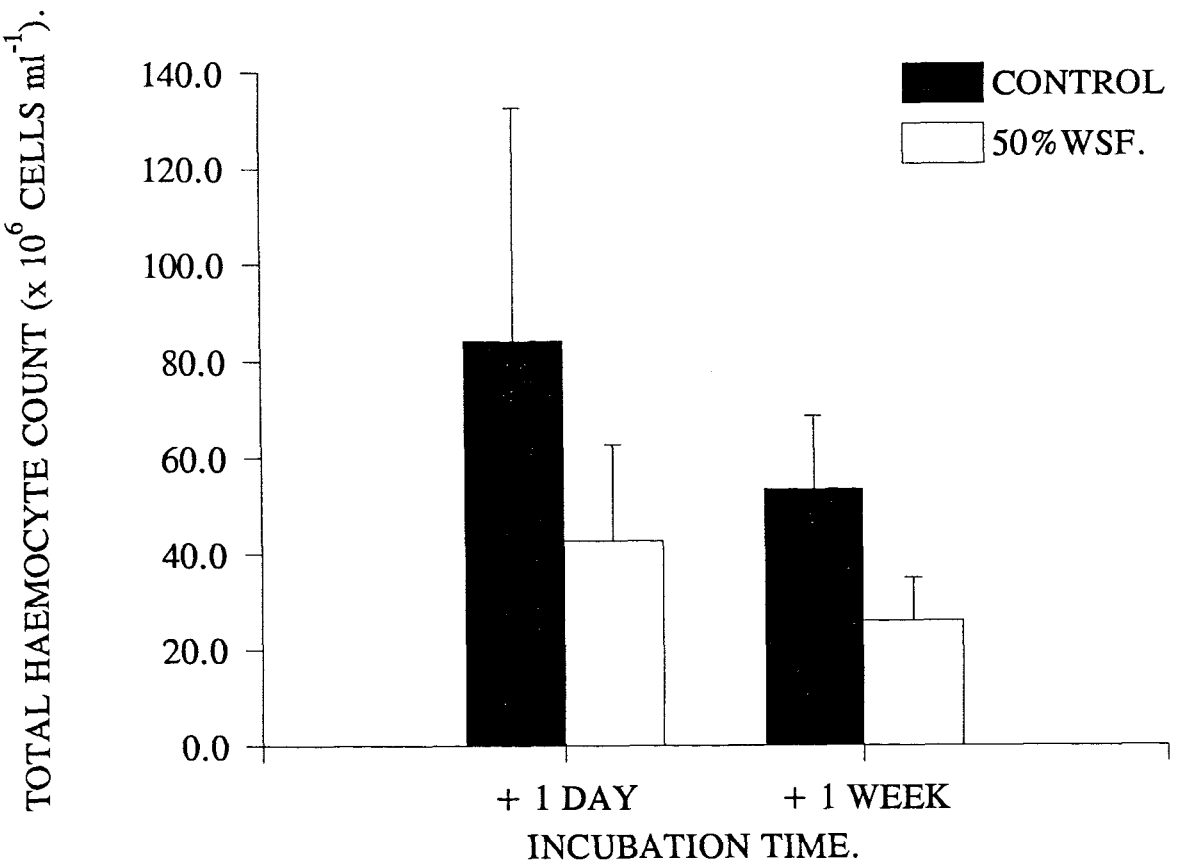

FIGURE 5.09 Fluctuations in the haemocyte population following hydrocarbon exposure in *C. maenas* as summarised in table 5.03, which also indicates the sample sizes.

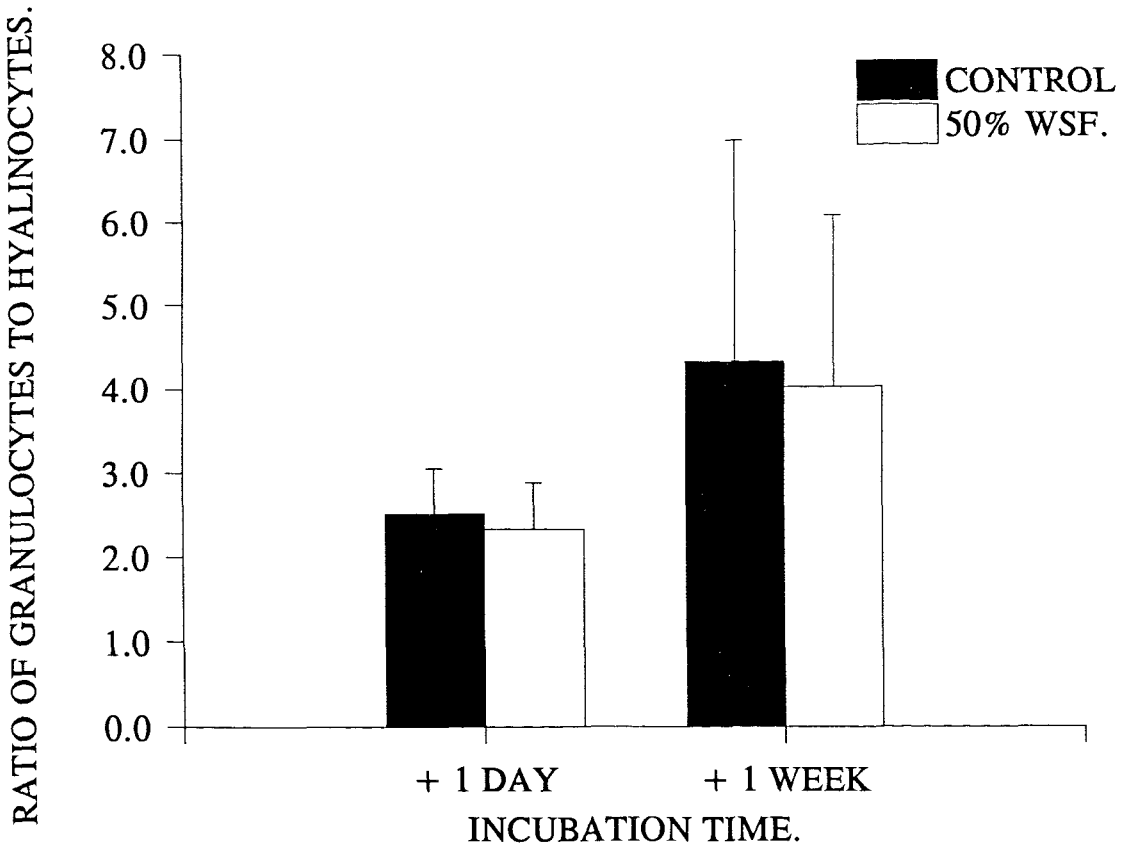

FIGURE 5.10 Changes in the haemocyte composition of the circulating haemocyte population calculated using the data presented in table 5.03. Figure shows the mean ± one standard deviation of the collected data, N = 6.

INCUBATION PERIOD	CONTROL		POLLUTED	
	HLS ENZYME ACTIVITY (UNITS. mg^{-1} PROTEIN)	N	HLS ENZYME ACTIVITY (UNITS. mg^{-1} PROTEIN)	N
+ 1 DAY	83.35 ± 67.06	6	75.60 ± 43.74	6
+ 1 WEEK	40.33 ± 62.82	6	188.37 ± 152.56	6

TABLE 5.04 Changes in haemocyte lysate supernatant (HLS) phenoloxidase enzyme activity (trypsin activated samples only) following incubation with 50% WSF. All values represent the mean ± one standard deviation of N observations.

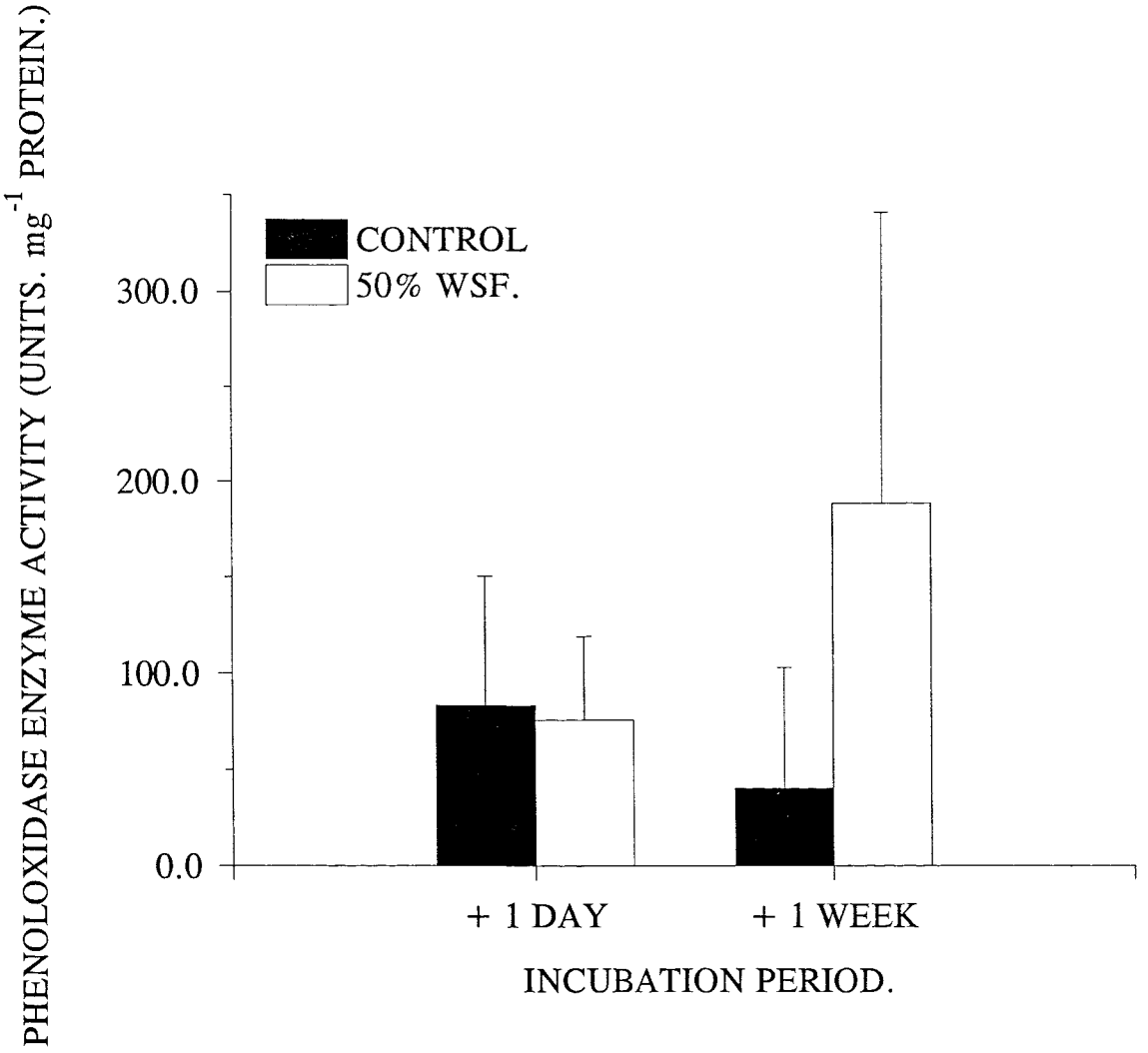

FIGURE 5.11 Effect of hydrocarbon exposure on the activity of the phenoloxidase enzyme as discussed in the results (section 5.3.2) showing the mean ± one standard deviation of six observations.

and figure 5.11. As discussed in chapter three the control activity was always very low and, rather than discuss these data separately, the control activity has been subtracted from the activated HLS and this corrected activity has been presented. From table 5.04 and figure 5.11 the extreme individual variability discussed in previous chapters is once again evident. This large degree of variability may in part explain why none of the statistical tests attempted proved to be significant. Having said this it is evident from figure 5.11 that following a week's exposure to 50% WSF there was a marked increase in the activity of the phenoloxidase enzyme (from 75.60 $\pm$ 43.74 units per mg. of protein to 188.37 $\pm$ 152.56 units per mg. protein). By comparison the activity in the control crabs decreased to approximately half of its starting value during the seven day incubation (decreasing from 83.35 $\pm$ 67.06 to 40.33 $\pm$ 62.82 units per mg. protein).

5.3.4 NEUTRAL RED RETENTION ASSAY.

The *in vitro* assay of neutral red retention was developed to investigate changes in cell stability following exposure to oil pollution. The release of neutral red (NR) following two and a half hours of dye loading has been indicated in figure 5.12 which shows the natural logarithmic transform of the collected data twenty-four hours after initial exposure. The data for one week after initial exposure have been summarised in figure 5.13, the regression lines describing the data have been plotted in both figures and the equations are presented in table 5.05. From table 5.05 and figures 5.12 and 5.13 it can be seen that the rate of NR loss increased as a function of the duration of the incubation. NR was lost at a faster rate in both the control and exposed animals at the end of the incubation than at the start. The data collected also indicate that the rate of NR loss was slightly faster (slope = - 0.006) in the 50% WSF exposed crabs a week after initial exposure than for the control animals (slope = - 0.002) held in experimental chambers for the same time. As indicated in table 5.05 however, none of these regression relations proved to be significant and the changes in rate of NR loss are only very slight.

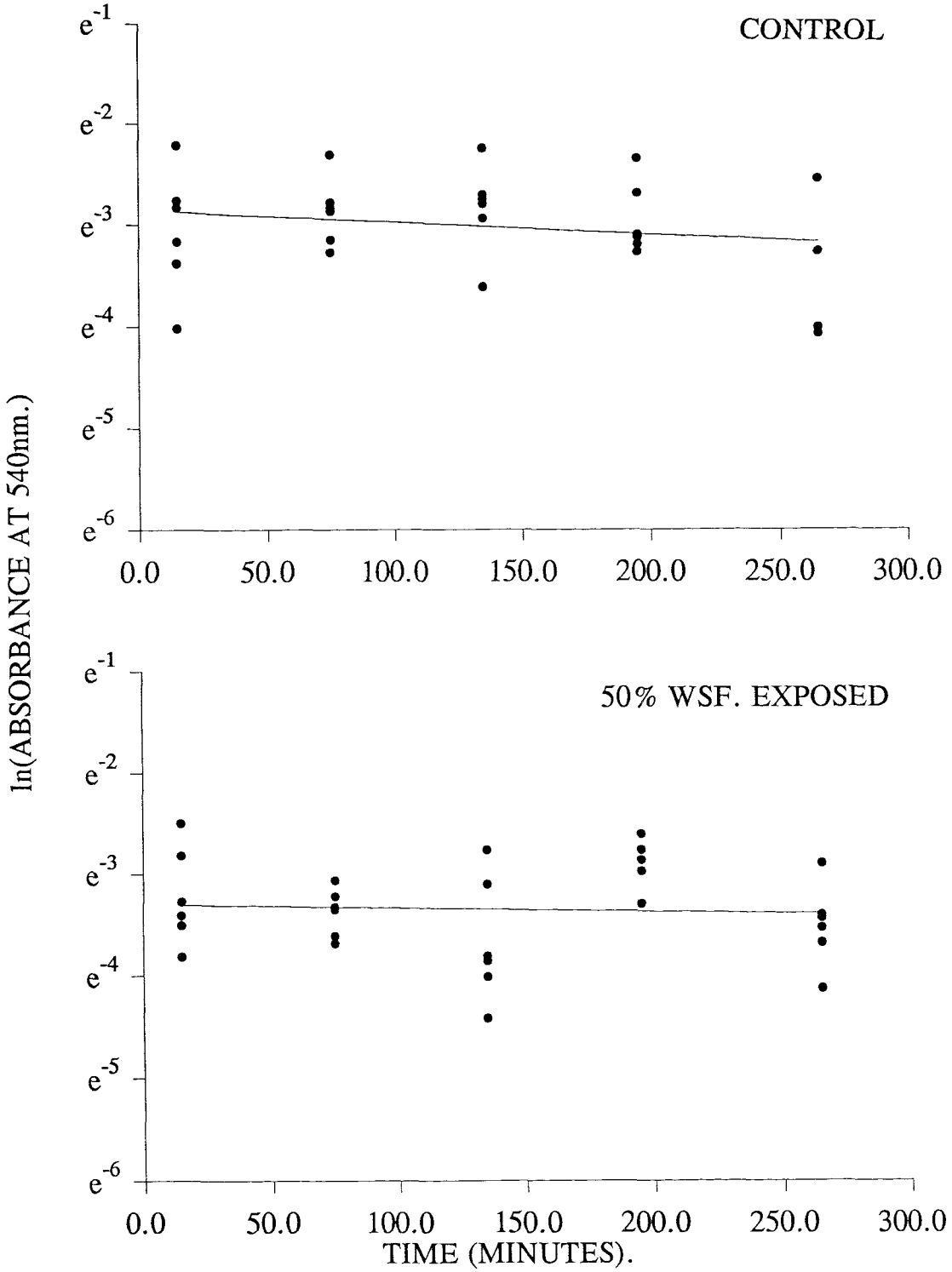

FIGURE 5.12 Rate of neutral red release twenty-four hours after initial exposure to 50% WSF, showing the natural logarithmic transformation of the collected data. Regression statistics for the line presented have been summarised in table 5.05, N = 6.

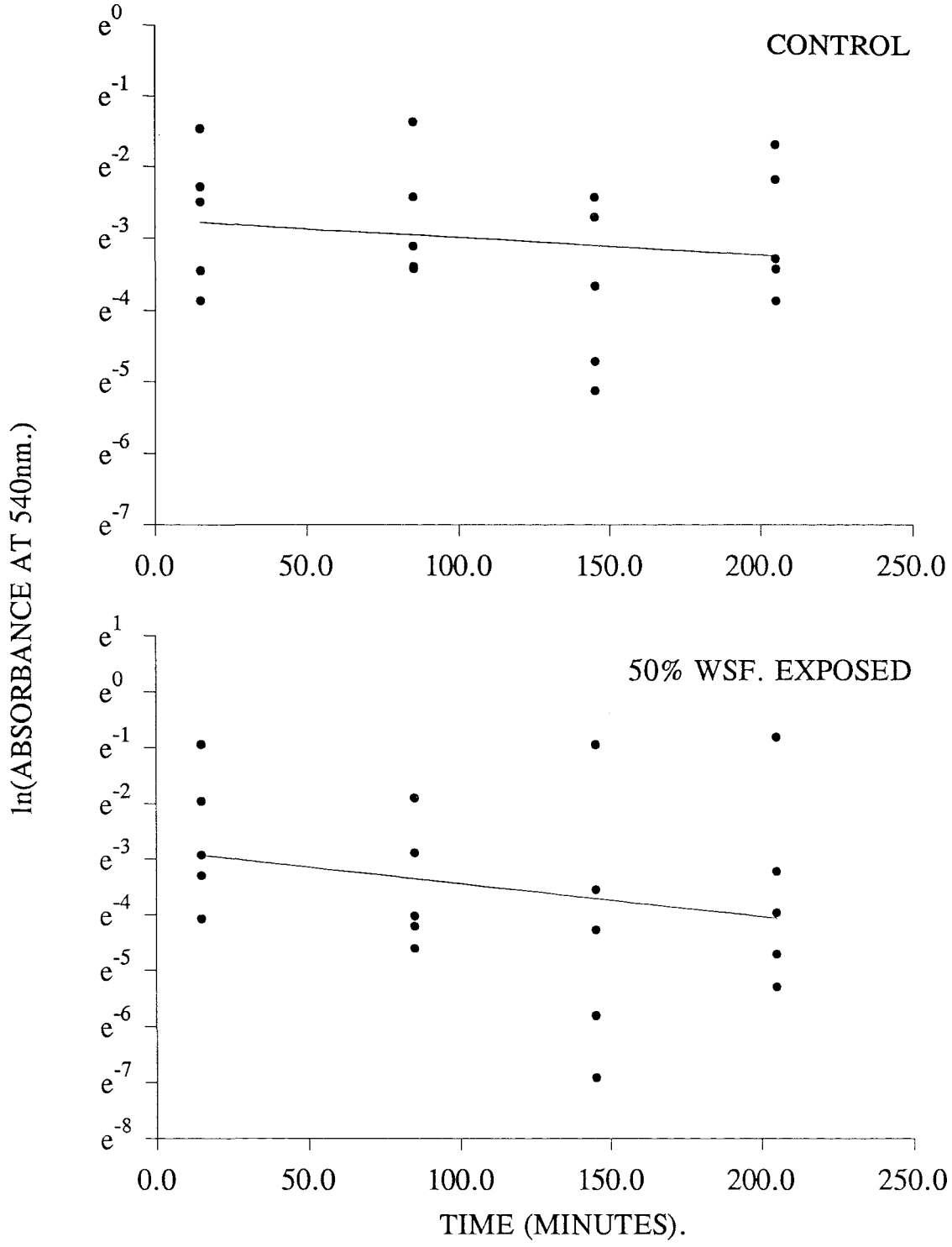

FIGURE 5.13 Rate of neutral red release from *C. maenas* haemocytes at the end of seven days incubation. Regression statistics have again been summarised in table 5.05, N = 6.

EXPERIMENTAL CONDITION	REGRESSION EQUATION	*P* VALUE	SIGNIFICANT ?
CONTROL + 1 DAY	ln (ABS) = (-1.21×10^{-3} TIME) - 2.85	0.296	NOT SIGNIFICANT
POLLUTED + 1 DAY	ln (ABS) = (-3.68×10^{-4} TIME) - 3.30	0.719	NOT SIGNIFICANT
CONTROL + 1 WEEK	ln (ABS) = (-0.002 TIME) - 2.74	0.464	NOT SIGNIFICANT
POLLUTED + 1 WEEK	ln (ABS) = (-0.006 TIME) - 2.84	0.261	NOT SIGNIFICANT

TABLE 5.05 Regression relations describing the rate of loss of neutral red from haemocytes as a function of incubation period and exposure condition. ABS = Absorbance at 540nm, ln = natural logarithmic function.

5.4 DISCUSSION.

This chapter presents an investigation into the consequences of a seven day exposure to a 50% dilution of the water soluble fraction (WSF) of Wyche Farm crude oil in *Carcinus maenas*.

The crude oil used in this study is of particular relevance to Southampton Water and the Solent region. It is drilled locally and transferred by pipeline to Southampton from where it is exported by tanker. Wyche Farm crude oil is a "light" oil with a large volatile fraction, being similar in nature to North Sea crude (Parkhouse, pers. comm.). The hydrocarbon concentration of the 100% WSF (150ppb) is in agreement with values previously determined for a range of commercially available oils. Anderson *et al.* (1974a) found that the WSF of the "heavy" Kuwait crude oil had a total hydrocarbon concentration of 78ppb whilst South Louisiana crude produced a water soluble fraction with a hydrocarbon concentration of 394ppb. The water soluble fractions of two refined oils analysed by Anderson *et al.* (1974a) were however, found to have much higher hydrocarbon concentrations (No. 2 fuel oil - 2002ppb; and Venezuelan bunker C oil - 935ppb.). The increase in the hydrocarbon content of the refined oils was shown to be a function of an increase in the concentration of the napthalenes and it is evident from these data that crude oils, such as the Wyche Farm Crude used in this study, have much smaller water soluble fractions than do the refined oils which have undergone some processing.

It must be borne in mind that for benthic marine invertebrates there are a number of possible routes by which hydrocarbon pollution can enter organisms and some of the previously cited workers have used oil contaminated sediment for studies of exposure on benthic fish and bivalves. *C. maenas*, whilst a member of the benthic community as an adult, has pelagic larval stages and further to this the gills and alimentary system of adult crabs (the sites of probable hydrocarbon uptake) are in contact with the polluted water column more than with contaminated sediment. Indeed, Anderson *et al.* (1974a) have concluded that estuarine animals are more exposed to oil-water mixtures and are ordinarily only in contact with those hydrocarbons in the aqueous phase, either in solution or as dispersed forms.

Savari (1988) extensively surveyed the sediments of Southampton Water and demonstrated that the non volatile hydrocarbons were accumulated to a concentration of two milligrammes per gramme of dry sediment. The aromatic component of the hydrocarbon load constituted approximately 30% with actual measured values varying between 235 and 591 microgrammes per gramme dry weight. The remaining non volatile hydrocarbons were shown to be mostly derived from the n-alkane series consisting of chains of sixteen to thirty carbon atoms. These molecules, by nature of their size, would be less toxic to organisms as they are only slowly absorbed. Water soluble fractions on the other hand tend to consist of small hydrocarbons such as the aromatics, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and short chain alkanes. These molecules tend to be more toxic as they are more readily absorbed by marine organisms. At the same time these compounds are volatile and Anderson *et al.* (1974a) have demonstrated that the toxicity of WSF decreases rapidly during the first twenty-four hours, losing as much as ninety percent of the original hydrocarbons. These workers demonstrated that the n-paraffins were lost to the atmosphere first followed by the aromatics. Atlas & Bartha (1972) have demonstrated that the loss of volatile components is accelerated at higher temperatures and as a consequence of aeration. For this reason the 100% WSF, once formulated, was *stored* at 5°C and in darkened bottles to reduce photo-oxidation and volatisation. The incubation chambers themselves were covered with glass sheets, mainly to prevent the animals escaping but also to prevent an excessive loss of hydrocarbons to the atmosphere. Temperature control was difficult during the incubation as the chambers were too big to fit into available water baths, however temperature fluctuations were limited by enclosing each chamber in a plastic bag which contained polystyrene chips.

The measured concentration of hydrocarbons in the WSF represents a value for a stored sample which would have undergone "limited weathering." Once the bottles were reopened and the fifty percent dilution of the WSF aerated the hydrocarbon concentration would have rapidly dropped over the first twenty-four hours (Atlas & Bartha, 1972) to a value of approximately 7.5ppb. Consequently the crabs would have been presented with short lived pulses of "concentrated"

hydrocarbons which would have reduced quite rapidly before being replaced every other day.

Perhaps the most striking observation of this study was the low concentration of all of the haemolymph sugars (between ten and thirty mg. per one hundred ml.) which were between 25% and 80% lower than those measured in the previous chapter which dealt with the consequences of a bacterial infection (figure 4.02, mean concentrations between forty and sixty mg. per one hundred ml.). It was suggested in chapter four that the high concentrations may have been a function of the stress associated with the infection and handling procedure (Telford, 1974b). With the present study it was not necessary to inject the crabs and, consequently, the organisms were handled less. It is therefore conceivable that this reduced disturbance was less stressful and the sugar concentrations were subsequently lower than in repeatedly handled individuals. This unverified statement is speculative and any comparisons between the two studies must be made with caution due to the differing nature of the experimental incubations. Whilst the temperature and salinity records for both studies were very similar the crabs were held in separate chambers during the bacterial infection and communally during the present investigation. Behavioural interactions between communally held animals may have caused an increase in locomotor activity in this study which would have led to an increase in oxidative metabolic processes consuming sugars. This second theory may also go some way to explain the lower haemolymph sugar concentrations in this present work.

It can be seen from figure 5.07 that a solution of 50% WSF produced very little obvious change in the carbohydrate status of *C. maenas*. The increase in total sugars in the hydrocarbon exposed crabs was mirrored by a similar increase in the control animals. This suggests that the increases observed may have been due to a common metabolic adaptation to continued starvation during the incubation. It is conceivable that the increase in measured sugar could have been due to the release of glycogen from a storage tissue such as the hepatopancreas (but see discussion in chapter two, section 2.4) into the haemolymph where upon it was metabolised to more available sub units. This theory is supported by an increase in the diversity

of the sugars towards the end of the week. If glycogen was metabolised it would have caused the observed increase in the haemolymph trehalose and also the possible increase in "other sugars" evident in figure 5.07. In chapter two it was demonstrated that an increase in haemolymph sugar correlated with a decrease in the hydrogen peroxide concentration during periods of low metabolic activity. Although no significant correlations were proved in this chapter it is evident by comparing figure 5.07 and figure 5.08 that as the sugar concentrations increased over the week incubation the hydrogen peroxide concentrations did fall. This again suggests that as the metabolic activity dropped for both the control and experimental crabs during the week, more unutilised carbohydrate remained in the haemolymph simultaneously reducing the concentration of metabolic end product hydrogen peroxide. From the statistical tests performed (*t* tests) it appears that the only effect hydrocarbon exposure had on the metabolic status of *C. maenas* was to accelerate this decline in metabolic activity. After a week incubation there was significantly more "unused" glucose in the haemolymph of the exposed crabs and significantly lower concentrations of the metabolic end product hydrogen peroxide.

In chapters three and four significant positive correlations between the concentration of the glucose dimer, trehalose and hydrogen peroxide were demonstrated. In both cases it was suggested that the trehalose may have been acting as a "sponge" for roaming superoxide radicals; however, no such correlation was proved in this present investigation. This observation does not however necessarily disprove the theory suggested from the previous two chapters. Superoxide production has only been demonstrated in *Carcinus maenas* following stimulation of the hyalinocytes with soluble bacterial mimics such as lipopolysaccharide from *Escherichia coli* and phorbol 12-myristate 13-acetate (PMA), which presumably would not have occurred in this instance.

Reductions in the metabolic activity, evidenced by a fall in the respiration rate, following hydrocarbon exposure have been demonstrated in marine invertebrates before. Anderson *et al.* (1974a) demonstrated reduced metabolic activity in *Palaemonetes pugio* and *Penaeus aztecus* exposed to a variety of water soluble fractions, similar observations were recorded by O'Hara *et al.* (1982) for *Carcinus maenas* and Sabourin & Tullis (1982) for the mussel, *Mytilus*

californicus. These observations are complimented by those of Heitz *et al.* (1974) who reported a reduction in some metabolic enzymes including: acid phosphatase, β glucuronidase, alkaline phosphatase, and glutamic-oxaloacetic transaminase, in shrimps (*Penaeus spp.*), oysters (*Crassostrea virginica*), and mullet (*Mugil cephalus*) on exposure to crude oil.

To conclude, it would seem that both exposed and control crabs exhibited a reduction in metabolic activity, evidenced by an increase in haemolymph sugar and a reduction in hydrogen peroxide, probably as a result of continued starvation through the incubation period. For the exposed crabs it is apparent that these changes had a quicker onset than for the control animals, producing significant differences between the start and end of the incubation.

A rapid reduction in the number of circulating haemocytes was seen following exposure to 50% WSF. Twenty-four hours after the initial exposure the haemocyte density was 51% of the control animals and this discrepancy was maintained throughout the seven day incubation, being approximately 48% at the end of the study. It is apparent therefore that hydrocarbon exposure caused a rapid reduction in the haemocyte population which was maintained for an extended period. Reports of reduced haemocyte population following hydrocarbon exposure are scarce in the literature. The earlier work of Smith & Johnstone (1992), who demonstrated a reduction in the haemocyte counts of the shrimp (*Crangon crangon*) following exposure to polychlorinated biphenyls (PCBs) at a concentration of 0.5 μgrammes per litre, represents the only reports found to date specifically dealing with marine crustaceans. More extensive, but at the same time more conflicting, reports have been made for marine bivalves. Fries & Tripp (1980) demonstrated haemocyte lysis following exposure of the clam *Mercenaria mercenaria* to concentrations of phenol greater than 10ppb, an observation which was supported by McCormick & Ray (1987) who demonstrated reduced total haemocyte counts in the mussels (*Mytilus edulis*) exposed for four weeks to an emulsion of 740μg. of oil. Conflicting data were reported by Balouet & Poder (1985) who found elevated haemocyte counts in bivalves collected from an oil spill site and inconclusive data was indicated by Anderson (1981) who found no

significant changes in the total or differential haemocyte counts of *Mercenaria mercenaria* exposed to sublethal doses of pentachlorophenol (PCP) or hexachlorobenzene (HCB). It would seem from these observations that there is a selective cytotoxicity of chemicals for certain haemocyte classes (Anderson, 1993) requiring careful analysis of individual cases.

A general decline in haemocyte number in both the control and experimental animals was superimposed on this hydrocarbon effect, possibly as a function of the absence of any food intake. Twenty-four hours after the incubation started there were approximately two and a half times as many granulocytes as there were hyalinocytes, by the end of the incubation this had increased to four granulocytes for every hyalinocyte in both the control and exposed crabs. This suggests that there was no influence of hydrocarbon exposure on the haemocyte composition but that there was a preferential removal of hyalinocytes from circulation during the incubation, possibly as a function of starvation.

Consideration must now be given to the fate of the removed haemocytes. It is possible that both the granulocytes and hyalinocytes may have lysed in the hydrocarbon-exposed crabs either as a consequence of changes in the osmotic potential of the plasma, following an influx of aromatic compounds into the haemolymph; or due an undefined detoxification mechanism (Fries & Tripp, 1980). However, cell lysis would have followed a decrease in cell membrane stability which should have been seen as changes (namely an increase) in the rate of neutral red release from the hydrocarbon exposed cells. It can be seen from figure 5.13 however that no obvious differences in the rate of NR release were recorded between the control and experimental crabs and as a result this theory can be rejected. It must also be remembered that extensive lysis of circulating haemocytes would have resulted in large amounts of cell debris being found in the haemocytometer chambers which was not observed during this study (unpubl. obs.).

A second possible explanation is that the haemocytes were sequestered within the gill filaments in a similar manner to that seen following the bacterial infection. Khan (1995) has reported inflammatory responses in gill tissue of the winter flounder (*Pleuronectes americanus)* following exposure to sediment

contaminated with Hibernia crude oil. Khan (1995) observed extensive hyperplasia of the gill filaments and excessive mucus production which led him to suggest the possibility of respiratory dysfunction causing mortality. It is conceivable that a similar response was elicited in this instance.

A third possibility is that continued exposure to hydrocarbon pollution would have caused excessive gill inflammation and eventually tissue necrosis and degeneration. Haemocytes would have been retained within the gill filaments as an immune response to this tissue breakdown as has been demonstrated previously for the clam (*M. mercenaria*) exposed to > 1ppb phenol (Fries & Tripp, 1976).

Finally it is conceivable that the removal of haemocytes from general circulation could have been a function of osmoregulatory requirements. Roesijadi & Anderson (1974) have previously demonstrated that hydrocarbon exposure (Prudhoe Bay crude oil contaminated sediment) led to a reduction in the free amino acid pool in the clam (*Macoma inquinata*). In chapter three it was suggested that haemocytes may have a role in augmenting the free amino acid pool in *C. maenas* and it is possible that following hydrocarbon exposure the haemocytes may have replenished a reduced amino acid pool, reducing any osmotic stress, in the posterior gills which are sites of osmoregulation in *C. maenas* (Taylor & Taylor, 1992; Pequeux, 1994).

These four suggested theories are, at best, speculative and would require validation by further experimentation, including extensive histological analysis, which was not possible within the present study. The radiolabelling of specific amino acids would also offer a potential method of clarifying these ideas.

Phenoloxidase enzyme activities were again very variable in this investigation and as a result none of the statistical comparisons proved to be significant. Having said this it can be seen from figure 5.11 that after one week's exposure to 50% WSF there were increases in some of the crab's enzyme activities. In light of the data presented in figure 5.09 it can be concluded that for those animals demonstrating an increase in enzyme activity the remaining granulocytes would have been very densely packed with prophenoloxidase. The increase in phenoloxidase seen in some crabs would, at first, appear to be

anomalous as Smith & Johnstone (1992) have previously reported that exposure of the shrimp (*Crangon crangon*) to hydrocarbons caused a reduction in phenoloxidase activity. These workers did however indicate that this inhibition of phenoloxidase was congener specific, PCB 15 (4,4'-dichlorobiphenyl) caused a reduction in enzyme activity whilst PCB 77 (3,3',4,4'-tetrachlorobiphenyl) elicited no response. Water soluble fractions of crude oil contain a vast array of hydrocarbon species that include aromatics, alkanes, and some polychlorinated biphenyls. It is possible that the WSF Wyche Farm crude oil does not contain the correct compounds to inhibit phenoloxidase activity.

Neutral red is a cytotoxic compound (Lowe & Pipe, 1994) which has been shown to produce changes in the structure of rat lysosomes as well as bringing about lysosomal enlargement and vacuolation (Ohkuma & Poole, 1981). As a consequence its use during the present study represents an additional stress to the cells; the dye retention time being a function of the stress imposed by the hydrocarbon exposure and the cytotoxic nature of neutral red. Neutral red retention (NRR) therefore represents a measure of the adaptive capacity of cellular processes following a prior exposure to contaminants (Lowe & Pipe, 1994).

In this instance neutral red release was determined to provide information on haemocyte stability and to possibly clarify whether or not the haemolymph sugar trehalose was involved in membrane stability (discussed at length in chapter four). It is evident from figure 5.12 and figure 5.13 however, that hydrocarbon exposure had very little influence on the stability of those haemocytes remaining in circulation. Indeed it was suggested above that, in light of these data, it was unlikely that the significant decreases in cell number seen for the hydrocarbon exposed crabs were due to cell lysis. Lowe *et al.* (1992) have demonstrated that measuring the rate of dye release from whole cells is not as sensitive as measuring the rate of release from individual lysosomes. Lowe *et al.* (1992) indicated that lysosomal neutral red retention times were reduced in the hepatocytes of dab (*Limanda limanda*) collected from polluted areas of the North Sea. It is possible that the usefulness of the NRR assay may be tissue dependent or simply that the sensitivity obtained by measuring whole cell stability was not sufficient for this

study. Attempts were made, whilst developing the NRR assay for crustacean haemocytes, to develop an assay to measure the rate of dye release from lysosomes within the haemocytes but problems were encountered as the cells proved extremely labile and readily lyased in tissue cultures held on microscope slides.

This present study has focused on the short and medium term effects of exposure to 50% WSF Wyche Farm crude oil, whilst some of the earlier work cited above has dealt with much longer incubations. One constraint in this work was the time available, but at the same time an incubation period of seven days was favoured as it was consistent with the bacterial infection study (chapter four). It was also necessary to limit the duration of the study and so prevent extended periods of starvation unduly influencing the measured indices; having said this the influence of no dietary intake is apparent in the data.

With this study males (over fifty-five millimetres carapace width) were used to avoid any sex or size linked differences in metabolism or immunology. It is well known however (Thorson, 1950; Lockwood, 1968; and Struhsaker *et al.*, 1974), that the breeding and larval stages of marine invertebrates are the most sensitive to environmental perturbation. Had sufficient time been available it would have proved interesting to investigate their responses hydrocarbon exposure.

To conclude it can be stated that there was a limited metabolic response to hydrocarbon exposure evident as a reduction in metabolic activity superimposed on a general decline due to continued starvation. Further to this there was a rapid reduction in haemocyte number which was maintained throughout the exposure probably representing a comprising of the crabs' immune systems; the phenoloxidase data however, proved equivocal. With these observations in mind it would seem that comparative haemocyte counts between control and experimental crabs are probably the most sensitive indicators of the stresses associated with hydrocarbon pollution. The limitation of isolated laboratory exposures to single stressors has repeatedly been pointed out throughout this thesis, and having defined the effects of bacterial infection and hydrocarbon exposure separately the effects associated with a synergistic exposure must now be considered.

CHAPTER SIX

EFFECT OF A SYNERGISTIC EXPOSURE TO HYDROCARBON POLLUTION AND PATHOGENIC INFECTION IN *Carcinus maenas*

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EFFECT OF A SYNERGISTIC EXPOSURE TO HYDROCARBON POLLUTION AND PATHOGENIC INFECTION IN *Carcinus maenas*

6.1 INTRODUCTION.

In comparison with the wealth of data published regarding the stresses imposed on marine organisms by heavy metal pollution, hydrocarbon pollution, and bacterial infection acting in isolation very few workers have investigated the more realistic, but inherently more complex effects of simultaneous exposure to a number of stressors. Occasionally, the effect of pollutant exposure on the *subsequent* immunocompetence of marine organisms has been alluded to (see reviews by Sindermann, 1993; and Anderson, 1993), but "cause and effect" has not always been implied. For example, Young & Pearce (1975) have reported a reduction in benthic macrofauna in the proximity of sewage sludge dumping sites in the New York Bight and also recorded an increased incidence of skeletal erosions, lesions, and ulcerations as well as black gill disease in both lobsters (*Homarus americanus*) and crabs (*Cancer irroratus*) collected from the dump sites. Although these observations may imply some form of interaction, it can be argued that they *prove* nothing. Indeed a recent ICES workshop concluded (Anonymous, 1985) that, "an established link between diseases and pollution does not exist;" although this view has been regarded as excessively conservative and negative by Sindermann (1993).

Most research adopting synergistic exposures of marine organisms to pollution and pathogenic infection has dealt with marine and freshwater fish species. In terms of non-hydrocarbon pollution Baker *et al.* (1983) have demonstrated an increase in the susceptibility of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Salmo gairdneri*) to infection by *Listonella*

anguillarum following a sub-lethal exposure to approximately 3.5 milligrammes of copper per litre, although the later work of Hetrick *et al.* (1984) found there to be no influence of exposure to chlorine (0.05 - 0.23 milligrammes per litre) on the susceptibility of the striped bass (*Morane saxatilis*) to *L. anguillarum* infection.

With respect to hydrocarbon-based pollution Fries (1986) has indicated a reduction in the number of plaque forming cells in specimens of the killifish (*Fundulus heteroclitus*) fed with a polychlorinated biphenyl (PCB) contaminated diet. Similarly Khan (1991) recently demonstrated that the survival of the winter flounder (*Pseudopleuronectes americanus*) exposed to both sediment contaminated with Hibernia crude oil and the haemoprotozoan parasite (*Trypanosoma murmarensis*) was reduced relative to fish exposed to either stressor in isolation. Khan (1991) explained his observation by citing Pickering & Pottinger (1989) who demonstrated that, in fish, chronic environmental stress raised levels of cortisol which, in turn, suppressed the immune system predisposing the fish to infection. More recently, Arkoosh *et al.* (1994) have reported that intraperitoneal injection of either 7,12-dimethylbenz-α-anthracene (DMBA) or the commercial PCB mixture Aroclor 1254 caused suppression of B cell mediated immunity in the chinook salmon (effective at doses of 0.6 milligrammes per kilogramme of salmon (DMBA), and 54 milligrammes per kilogramme (Aroclor 1254)). Further to this, they reported that there was a decrease in the titre of antibodies to the *Listonella anguillarum* O antigen in rainbow trout (*Onchorynchus mykiss*) fed a PCB contaminated diet.

Of more interest to the present research on *Carcinus maenas* are those studies dealing with immune systems that are not reliant on molecules such as the immunoglobulins and the cortisol steroids. Jefferies (1972) has demonstrated that pollution exposed clams (*Mercenaria mercenaria*) have a higher incidence of *Polydora* infestation; observations that were later supported by Anderson et al. (1981) who exposed *M. mercenaria* to the three hydrocarbons: benzo-α-pyrene, pentachlorophenol (PCP), and hexachlorobenzene (HCB), for eighteen weeks after which time the animals demonstrated an inability to clear the Gram negative bacteria *Flavobacterium* spp. from their haemolymph. For the Crustacea Couch & Courtney (1977) recorded an increased incidence of *Baculovirus* infections in

pollution exposed shrimp in the laboratory concluding that the pollution alone led to the observed immunosuppression and Nimmo *et al.* (1978) have demonstrated atypical secondary bacterial infections in cadmium exposed shrimp (*Penaeus duorarum*, *Palaemonetes pugio*, and *Palaemonetes vulgaris*). The data presented in this study (chapter five) also suggest that there may be immunosuppression following hydrocarbon exposure. Following exposure of *C.maenas* to 50% WSF Wyche Farm crude oil a rapid decline in the haemocyte population was seen which was maintained throughout the incubation period.

It is apparent from the work discussed above that the stresses associated with pollution tend to have a suppressive effect on the immunocompetence of marine organisms. This chapter summarises an investigation into the metabolic and immunological consequences of a simultaneous exposure to hydrocarbon pollution (50% WSF) and bacterial infection (*L. anguillarum*) in the shore crab, *Carcinus maenas*. A simultaneous exposure was adopted instead of the sequential exposures of Anderson *et al.* (1981); Baker *et al.* (1993); and Hetrick *et al.* (1994) as the consequences of a synergistic challenge on the condition of C. maenas were of interest, rather than the consequences of hydrocarbon exposure on the susceptibility to a *subsequent* bacterial infection. Further to this it was hoped to avoid prolonged periods of starvation, the effects of which were apparent during previous incubations as discussed in chapters four and five, and also to prevent any possibility of adaptation to the pollution stressor before injection with *Listonella anguillarum*.

6.2 METHODS.

6.2.1 COLLECTION, MAINTENANCE AND GENERAL EXPERIMENTAL PROCEDURE.

Crabs were collected from the salt marsh system around Hayling Island and acclimated to the experimental conditions of: temperature 23.4 ± (SD) 1.3°C and salinity 33.8 ± (SD) 1.4‰; in the dark as summarised in chapter four. In accordance with all previous studies only intermoult male crabs, of greater than fifty-five millimetres carapace width, were used to avoid any sex or size linked differences in metabolism and immunology (see section 2.2.1).

This synergistic investigation was conducted over a four week period from 22nd June to 20th July 1995. During the first week the responses of the metabolic indices were determined followed by the haemocyte studies and phenoloxidase determinations in the second and third weeks. In the fourth and final week cellular stability was once again investigated using the neutral red retention assay developed in chapter four.

For each week (seven day) incubation two groups (six crabs per group) were used; one group of control animals were maintained in sterile aquarium seawater and were sham injected with sterile *Carcinus* saline according to the procedure detailed in chapter four (see section 4.2.1). The second group of six animals were subjected to solutions of 50% WSF formulated according to the protocol described in chapter five (section 5.2.2) except that any seawater used (either to produce the 100% WSF or to dilute it to 50% WSF) was first sterilised by autoclaving at 120°C for twenty minutes to remove any live pathogens. This second group of experimental animals was also infected with live inoculations of the bacterium *Listonella anguillarum*, grown according to the method outlined in section 4.2.1. To minimise the risk of cross infection both the control and experimental crabs were incubated in individual glass topped chambers in contrast to the communal chambers used in the hydrocarbon exposure study (see chapter five). The chambers were not of a flow through design and the incubation water was changed on alternate days to avoid the accumulation of toxins and to replenish

the hydrocarbon concentration in the exposed incubation (discussed in chapter five, section 5.2).

6.2.2 METABOLIC AND IMMUNOLOGICAL INDICES OF ORGANISM CONDITION.

All of the measured indices were assayed using the protocols described in chapter four (section 4.2.2). The neutral red retention assay used to measure cell membrane stability was the same as described in chapter five (section 5.2.3.1).

In chapter two the concept of plasma phenoloxidase enzyme activity was first suggested and it was briefly mentioned again in chapter five. Despite continued attempts during this research programme no suitable protocol was developed which could prove or disprove, with any certainty, the presence of active phenoloxidase in the plasma. Once collected in marine anticoagulant (pH = 4.6, Söderhäll & Smith, 1983) the haemolymph sample of *C. maenas* was acidic which would have inhibited the activity of any phenoloxidase present within the plasma. As a result once the plasma samples were collected a method had to be determined to enable their neutralisation.

Haemolymph samples were collected in marine anticoagulant as described in section 4.2.2 and immediately overlain with liquid paraffin to avoid the premature melanisation of the plasma (this represents an adaptation of White *et al.* (1985) and Hauton *et al.* (1995) who both suggested the use of silicon oil). Once the paraffin had sealed the samples they were centrifuged to sediment the haemocytes according to the procedure described in section 2.2.2.4. Two aliquots of four hundred microlitres each were drawn from each of the haemolymph samples and were neutralised with a variety of buffers and alkalis before one aliquot was incubated with trypsin (activated samples) whilst the second was incubated with cacodylate buffer (control samples) as described for the HLS samples in section 2.2.2.4. Both the activated and control samples were subsequently assayed for phenoloxidase activity using L-DOPA as a substrate (see section 2.2.2.4). Tests were initially performed using bromothymol blue pH indicator to ensure that the assays were correctly neutralised. At the same time it

was necessary to ensure that the neutralising agents used did not mimic the action of phenoloxidase by chemically driving the oxidation of L-DOPA to DOPAquinone. Unfortunately, all of the neutralising agents used proved unsuitable; a variety of buffers of increasing alkalinity were used but even the most alkali buffer (sodium carbonate/sodium bicarbonate, pH = 11.0) had to be added in such large volumes to the plasma sample that measuring enzyme activity proved impractical. A range of alkalis were also tried as alternatives and forty microlitre additions of solutions of 1M sodium carbonate and 1M sodium hydroxide successfully neutralised the assay (solutions of 2M potassium chloride and 3.2M ammonium sulphate were also tried, but again had to be added in too large a volume to make the assay worthwhile). However, both the sodium carbonate and sodium hydroxide drove the conversion of DOPA to DOPAquinone when tested in reagent blanks. Attempts were made to assay reagent blanks alongside plasma samples to ascertain whether any enzyme activity could be measured superimposed on the absorbance changes due to the alkalis added but this also proved inconclusive. As a consequence of these problems and due to the limitations of time attempts to measure plasma phenoloxidase had to be suspended in order to complete the synergistic incubation programme.

6.2.3 STATISTICAL ANALYSIS OF DATA.

The data collected from the synergistic incubations were analysed using *t* tests (with Mann-Whitney Rank Sum tests used as a non parametric alternative when the data were not normally distributed) as has been discussed in section 4.2.4. Significant differences were observed when $P < 0.05$.

6.3 RESULTS.

6.3.1 INDICES OF METABOLIC STATUS.

The changes in carbohydrate composition of *C. maenas* associated with a synergistic exposure to 50% WSF and sub-lethal infection with *Listonella anguillarum* have been summarised in table 6.01 and figure 6.01 from which it can be seen that there were significant (*t* tests, $P < 0.05$) increases in the total concentration of circulating sugars for both the control and experimental crabs during the seven day exposure period. A day after the incubation started total sugar concentrations were almost identical for the control and experimental crabs (35.00 ± 12.47 and 32.19 ± 7.77 mg. per one hundred ml., respectively) and these increased in both cases during the incubation to reach 60.72 ± 9.74 mg. per one hundred ml. (control, representing a 73.49% increase) and 48.05 ± 15.36 mg. per one hundred ml. (experimental, representing a 49.27% increase) by the end of the week.

From figure 6.01 it can be seen that these increases in the total sugar concentration were due, in part, to significant (*t* tests, $P < 0.05$) increases in haemolymph trehalose in both the control and experimental crabs over the week incubation (from 2.69 ± 1.18 mg. per one hundred ml. (control) and 2.68 ± 0.87 mg. per one hundred ml. (experimental) after one day to 4.38 ± 1.08 and 3.94 ± 1.03 mg. per one hundred ml. respectively after seven days incubation). However, the greatest contribution made to the circulating carbohydrate pool seems to have been from the "other sugars" which have been inferred from the difference between the concentration of total sugars and the combined concentration of the glucose and trehalose components. As shown in table 6.01 this component constituted approximately 50-70% of the total circulating sugar in the haemolymph throughout the incubation. In contrast it is evident that the glucose constituted a smaller proportion of the circulating sugar. From table 6.01 it can be seen that even though the glucose concentrations remained constant at approximately 11-12 mg. per one hundred ml. for both the control and experimental crabs throughout the entire study the percentage contribution made to the haemolymph sugar pool

+ 1 DAY.						
SUGAR	CONTROL			EXPERIMENTAL		
	mg. $100ml^{-1}$	%	N	mg. $100ml^{-1}$	%	N
TOTAL SUGAR	35.00 ± 12.47	100.00	6	32.19 ± 7.77	100.00	6
GLUCOSE	11.24 ± 5.15	32.11	6	12.74 ± 3.32	39.58	6
TREHALOSE	2.69 ± 1.18	7.69	6	2.68 ± 0.87	8.33	6
OTHER SUGARS	**21.07**	**60.20**	-	**16.77**	**52.09**	-
+ 1 WEEK.						
SUGAR	CONTROL			EXPERIMENTAL		
	mg. $100ml^{-1}$	%	N	mg. $100ml^{-1}$	%	N
TOTAL SUGAR	60.72 ± 9.74	100.00	6	48.05 ± 15.36	100.00	6
GLUCOSE	13.89 ± 9.33	22.89	6	11.91 ± 6.90	24.79	6
TREHALOSE	4.38 ± 1.08	7.21	6	3.94 ± 1.03	8.20	6
OTHER SUGARS	**42.45**	**69.90**	-	**32.20**	**67.01**	-

TABLE 6.01 Changes in the haemolymph sugar composition of *Carcinus maenas* during a synergistic incubation with 50% WSF and *Listonella anguillarum*. Table shows means ± one standard deviation of N observations. Figures in bold have been inferred as discussed in the text.

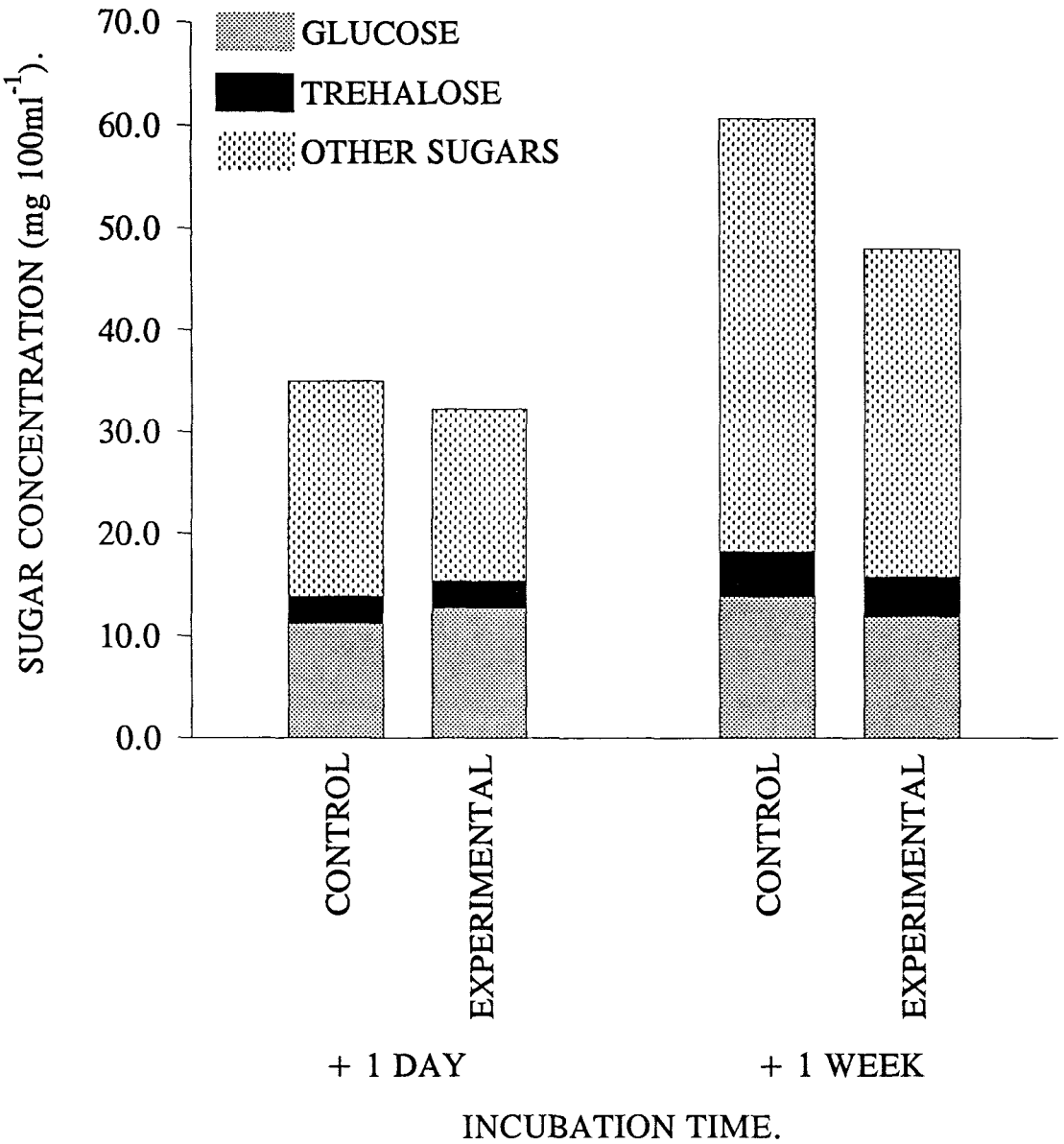

FIGURE 6.01 Changes in the haemolymph sugar composition of *Carcinus maenas* following exposure to oil pollution and bacterial infection. Data presented as summarised in table 6.01 with the "other sugar" component inferred as discussed in the text, N = 6.

INCUBATION PERIOD	CONTROL		EXPERIMENTAL	
	[HAEMOLYMPH HYDROGEN PEROXIDE] (mg. l^{-1})	N	[HAEMOLYMPH HYDROGEN PEROXIDE] (mg. l^{-1})	N
+ 1 DAY	30.33 ± 20.67	6	24.11 ± 12.91	6
+ 1 WEEK	13.70 ± 5.29	6	22.20 ± 13.15	6

TABLE 6.02 Changes in the concentration of haemolymph hydrogen peroxide as a consequence of synergistic exposure to 50% WSF of Wyche Farm crude oil and infection with *Listonella anguillarum*. All values represent the mean ± one standard deviation of N observations.

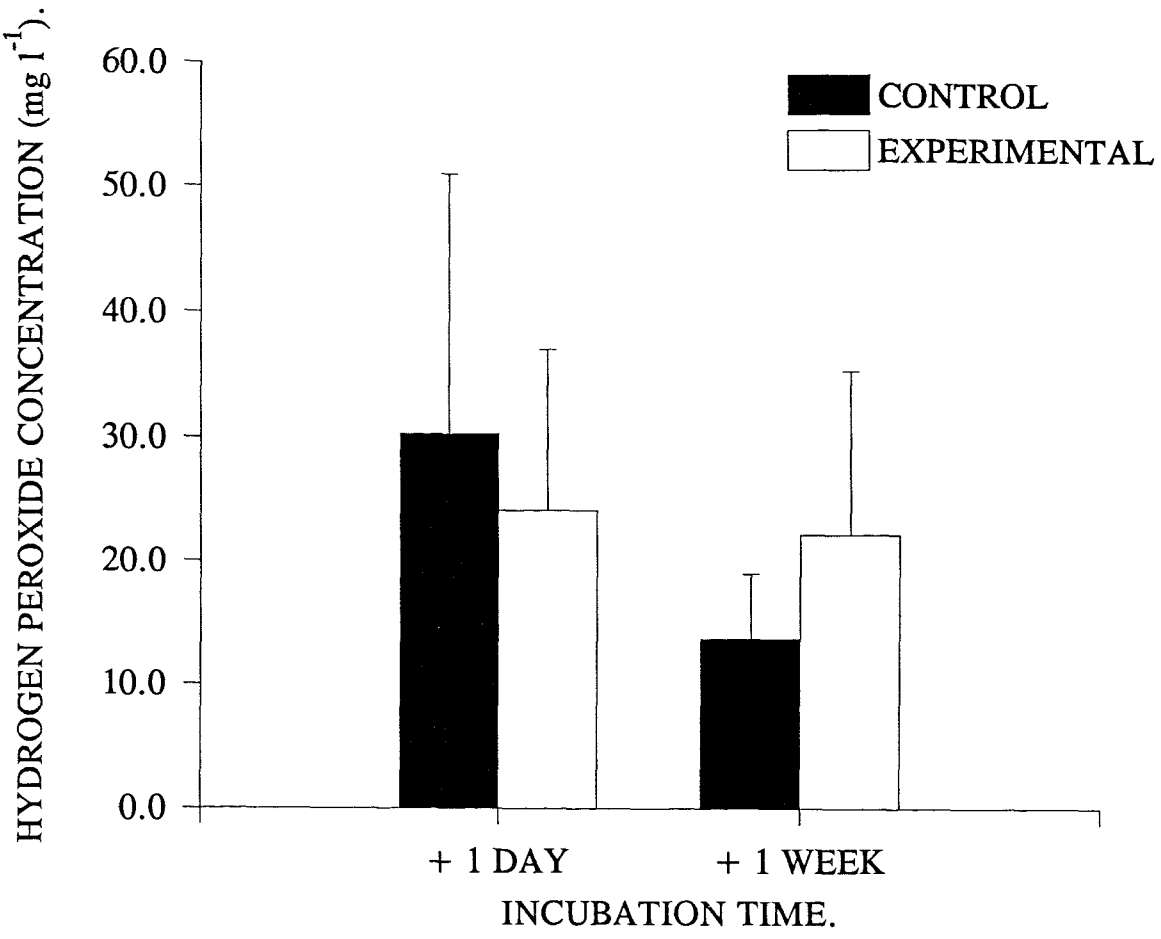

FIGURE 6.02 Changes in the concentration of haemolymph hydrogen peroxide in crabs exposed to 50% WSF and simultaneous infection with *Listonella anguillarum* showing the mean ± one standard deviation of six observations.

diminished to approximately 23% by the end of the incubation as a function of the increasing trehalose and "other sugar" components.

From the data summarised in table 6.02 and figure 6.02 it can be seen that the haemolymph hydrogen peroxide concentrations varied considerably between individual crabs giving rise to large standard deviations in the calculated means. As a consequence of this variation none of the statistical comparisons proved significant. Having said this however, it can be seen from figure 6.02 that both the control and experimental crabs demonstrated declining hydrogen peroxide concentrations during the week incubation and that this decline was more pronounced in the case of the control animals (dropping from 30.33 $\pm$ 20.67 mg. per litre to 13.70 $\pm$ 5.29 mg. per litre) than for the synergistically exposed crabs (24.11 $\pm$ 12.91 mg. per litre to 22.20 $\pm$ 13.15 mg. per litre).

Pearson Product Moment correlations were performed on the sugar and hydrogen peroxide data to investigate the possibility of any interactions between the variables as has been discussed in chapters four and five. In this instance however there were no significant positive or negative correlations between any of the variables measured.

6.3.2 INDICES OF IMMUNOCOMPETENCE.

It is evident from figure 6.03 that there were significant reductions in the total haemocyte count (THC) for both the control and experimental crabs in this incubation. Further to this it can be seen that the reduction in circulating haemocytes is greatest in the synergistically exposed crabs with the haemocyte counts approximately halving in value by the end of the incubation (from 95.16 $\pm$ 41.84 x 10^6 to 44.51 $\pm$ 28.44 x 10^6 cells per ml.). The data used to construct figure 6.03 have been presented in table 6.03 from which it can be seen that the reductions in THC were due to a decrease in both the circulating granulocytes and hyalinocytes in both control and experimental *C. maenas*. The granulocyte and hyalinocyte data have been summarised graphically in figure 6.04 from which it is evident that there were only small increases in the ratio of granulocytes to hyalinocytes for both the control and experimental crabs although neither of these

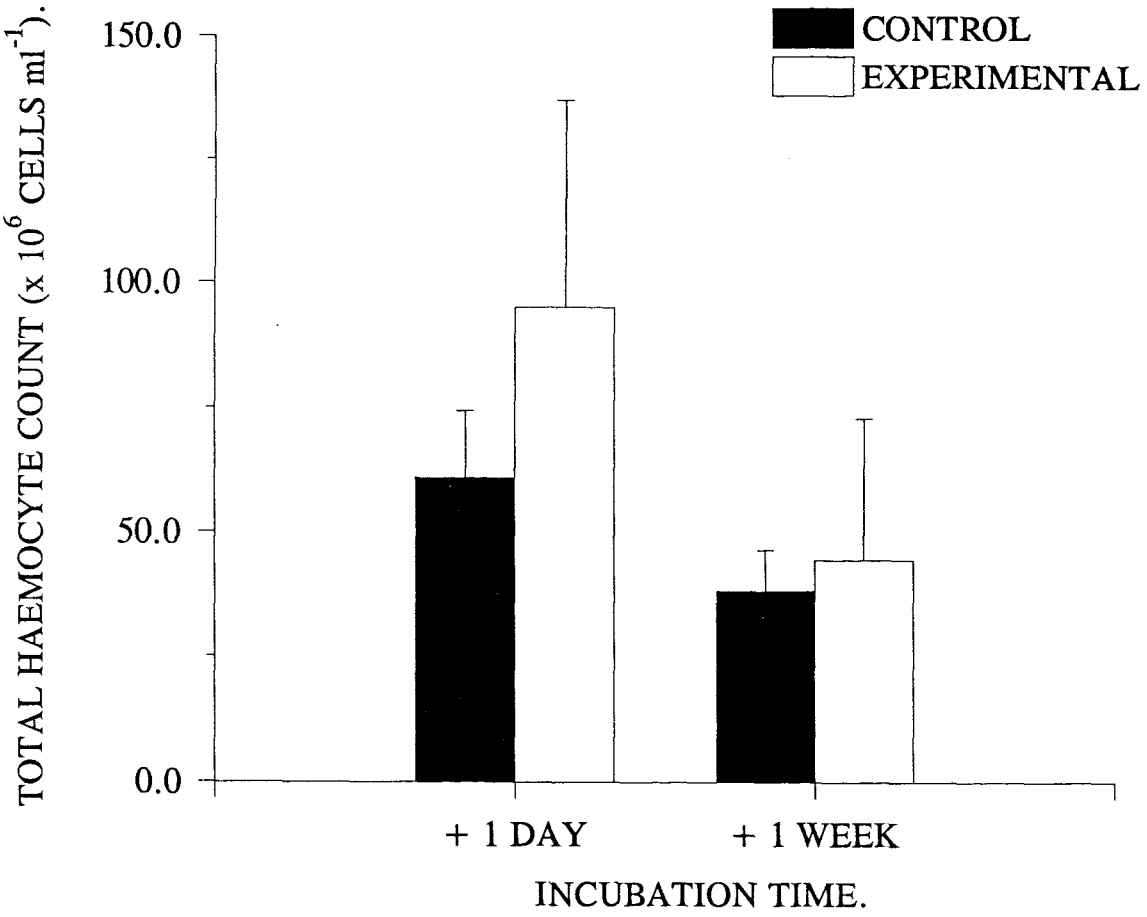

FIGURE 6.03 Fluctuations in the circulating haemocyte population in C. maenas following synergistic exposure to oil pollution and bacterial infection showing the mean ± one standard deviation of six observations as summarised in table 6.03

CONTROL CRABS.				
INCUBATION PERIOD	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
+ 1 DAY	43.24 ± 8.31	17.82 ± 6.65	61.06 ± 13.44	6
+ 1 WEEK	28.98 ± 7.44	9.30 ± 2.01	38.28 ± 8.27	6
EXPERIMENTAL CRABS.				
INCUBATION PERIOD	NO. GRANULOCYTES ($x10^6$ ml^{-1})	NO. HYALINOCYTES ($x10^6$ ml^{-1})	TOTAL HAEMOCYTES ($x10^6$ ml^{-1})	N
+ 1 DAY	71.93 ± 31.47	23.24 ± 10.60	95.16 ± 41.84	6
+ 1 WEEK	35.13 ± 23.29	9.38 ± 5.52	44.51 ± 28.44	6

TABLE 6.03 Summarising the affect of exposure to hydrocarbons and bacteria on the circulating haemocyte population during the week incubation. Table shows the mean ± one standard deviation of N observations.

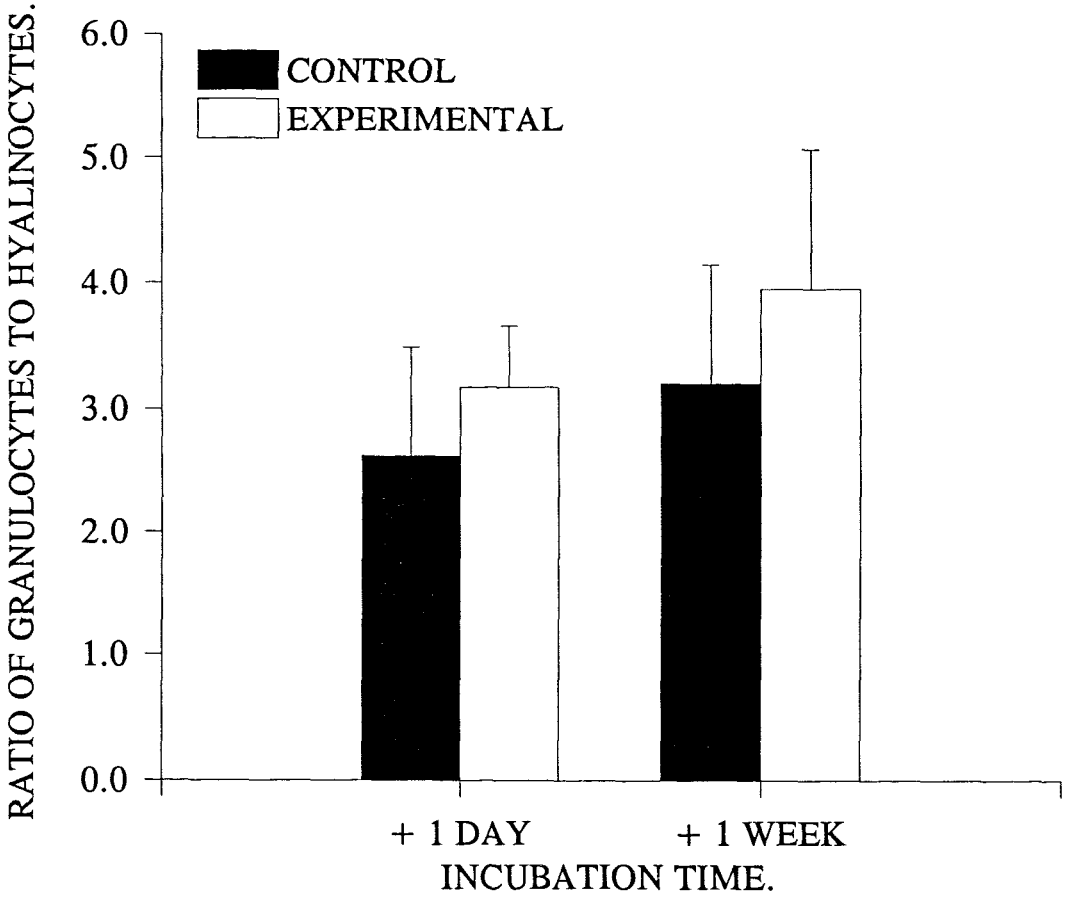

FIGURE 6.04 Changes in the composition of the circulating haemocyte population expressed as the ratio of granulocytes to hyalinocytes using the data presented in table 6.03. Figure shows the mean $\pm$ one standard deviation of six observations.

INCUBATION PERIOD	CONTROL		EXPERIMENTAL	
	HLS ENZYME ACTIVITY (UNITS. mg^{-1} PROTEIN)	N	HLS ENZYME ACTIVITY (UNITS. mg^{-1} PROTEIN)	N
+ 1 DAY	84.08 ± 56.01	6	85.42 ± 67.16	6
+ 1 WEEK	2.83 ± 3.08	6	1.78 ± 2.44	5

TABLE 6.04 Changes in haemocyte lysate supernatant (HLS) phenoloxidase enzyme activity (trypsin activated samples only) following synergistic incubation with 50% WSF and infection with *L. anguillarum*. All values represent the mean ± one standard deviation of N observations.

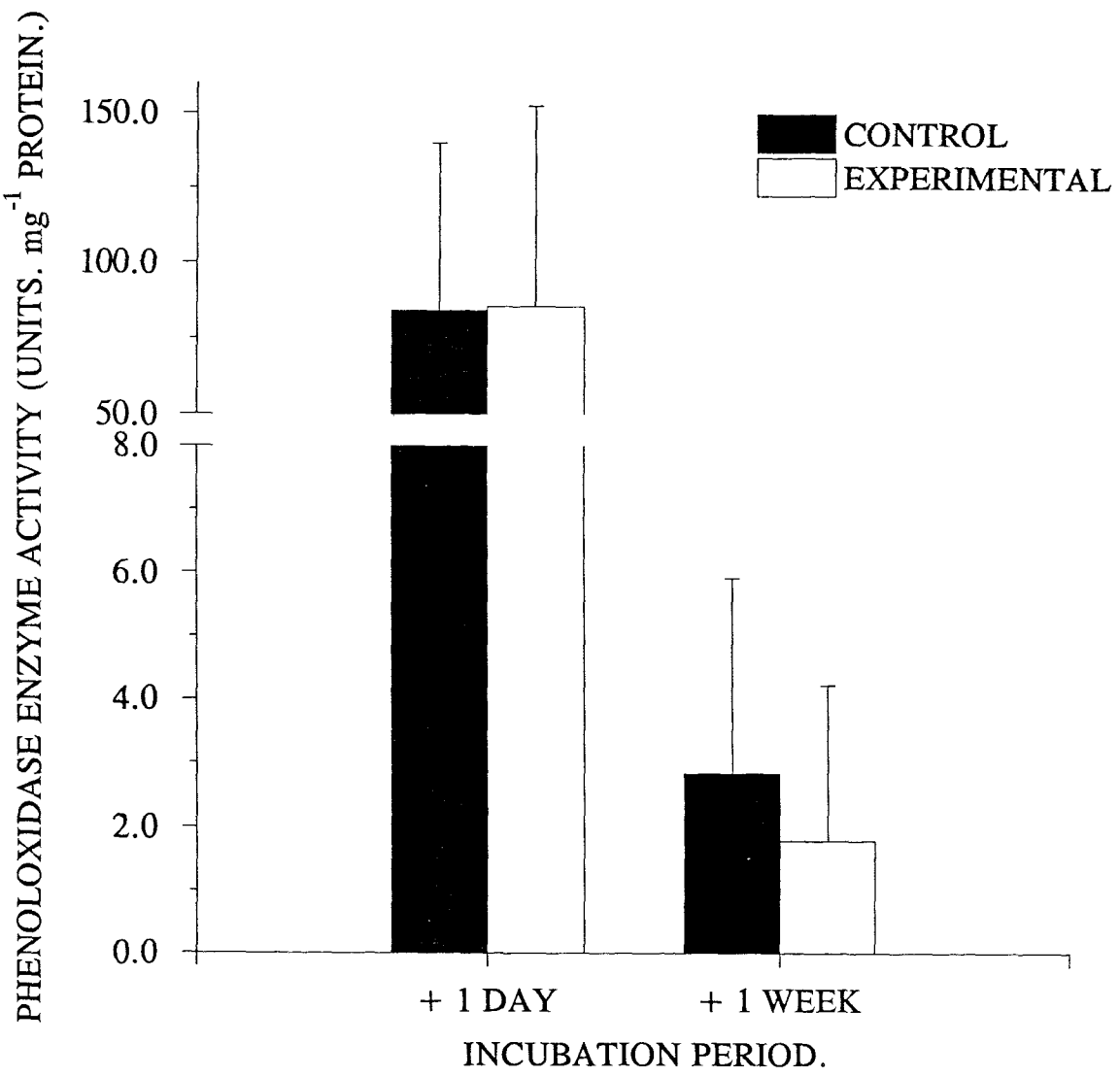

FIGURE 6.05 Phenoloxidase enzyme activities following synergistic exposure to a live *L. anguillarum* infection and 50% WSF Wyche Farm crude oil showing the mean $\pm$ one standard deviation of N observations. Note the axis break and change of scale between 8.0 and 50.0 units per milligramme of HLS protein.

increases proved significant ($P > 0.05$).

Changes in the activity of the phenoloxidase enzyme have been indicated in figure 6.05 and table 6.04 from which it can be seen that there were large and statistically significant reductions in the enzyme activity during the week incubation, decreasing from 84.08 ± 56.01 to 2.83 ± 3.08 units per mg. HLS protein in controls; and from 85.42 ± 67.16 to 1.78 ± 2.44 units per mg. HLS protein in the experimental crabs. Once again however there was no apparent difference between the control and experimental crabs at the beginning or end of the incubation. As can be seen from table 6.04 there were only five experimental crabs sampled at the end of the synergistic incubation. During the study one of the experimental crabs died and was removed from the experiment. This proved to be the only incidence of mortality in the synergistic investigation.

6.3.3 NEUTRAL RED RETENTION ASSAY.

The rates of neutral red release have been summarised in figure 6.06 for the day after the start of the incubation and in figure 6.07 for the end of the incubation. Table 6.05 summarises the regression relations for all of the collected data and from this it can be seen that a day after the start of the incubation the rates of dye release (slope of line) were significant and identical for both the control and experimental organisms. After a week's incubation the rate of dye release dropped in the control crabs (-1.42×10^{-3} units per minute, trend not significant) and also in the experimental animals (-0.004 units per minute, $P < 0.05$) from which it can be suggested that there was an increase in haemocyte stability in the control and experimental crabs and further to this that the increase in stability was more pronounced in the control animals.

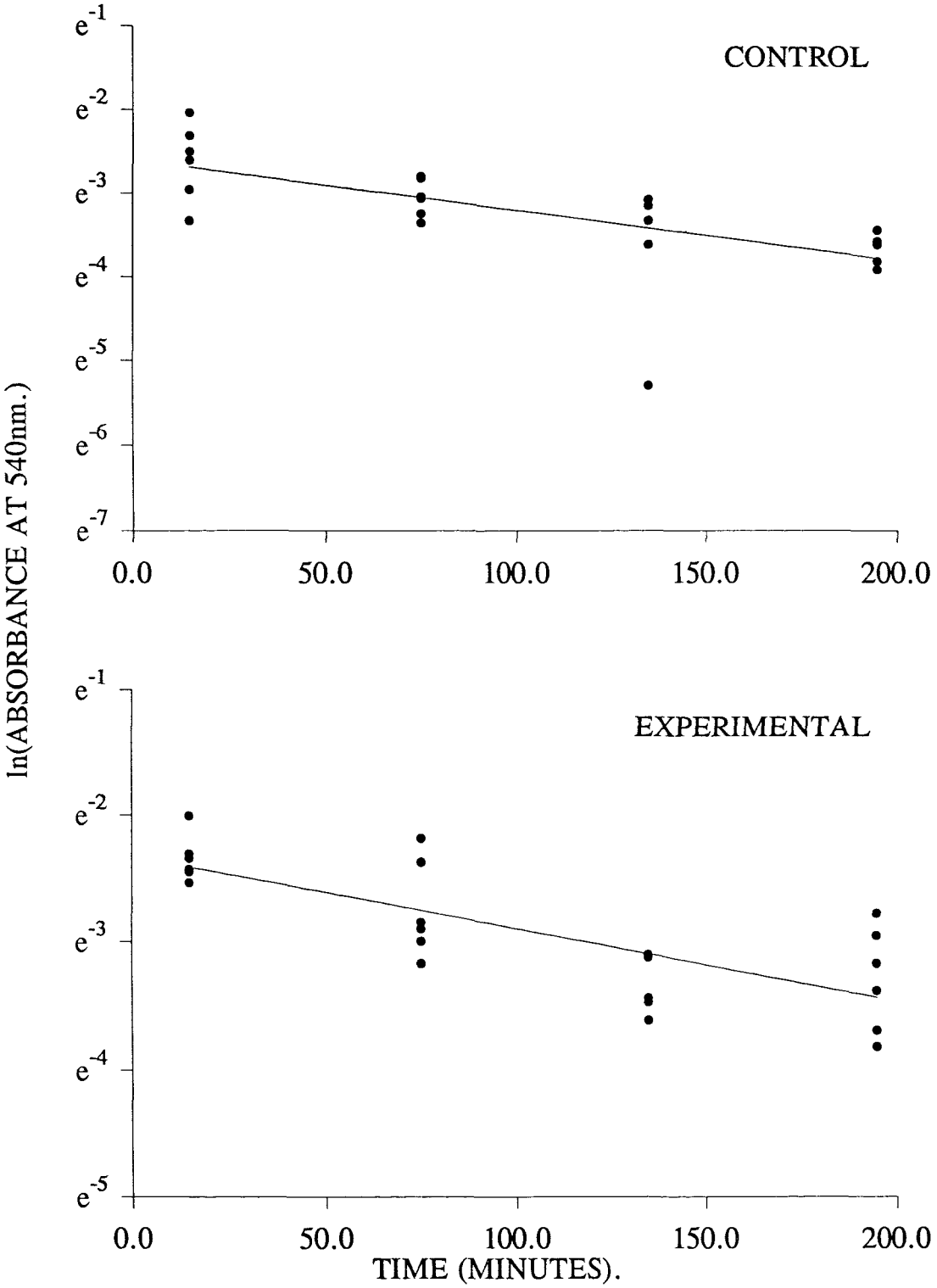

FIGURE 6.06 Rates of neutral red release after 2.5 hours of dye loading. Figure compares the data collected for the control and experimental crabs twenty-four hours after the start of the incubation. The regression statistics for the two plotted lines have been summarised in table 6.05.

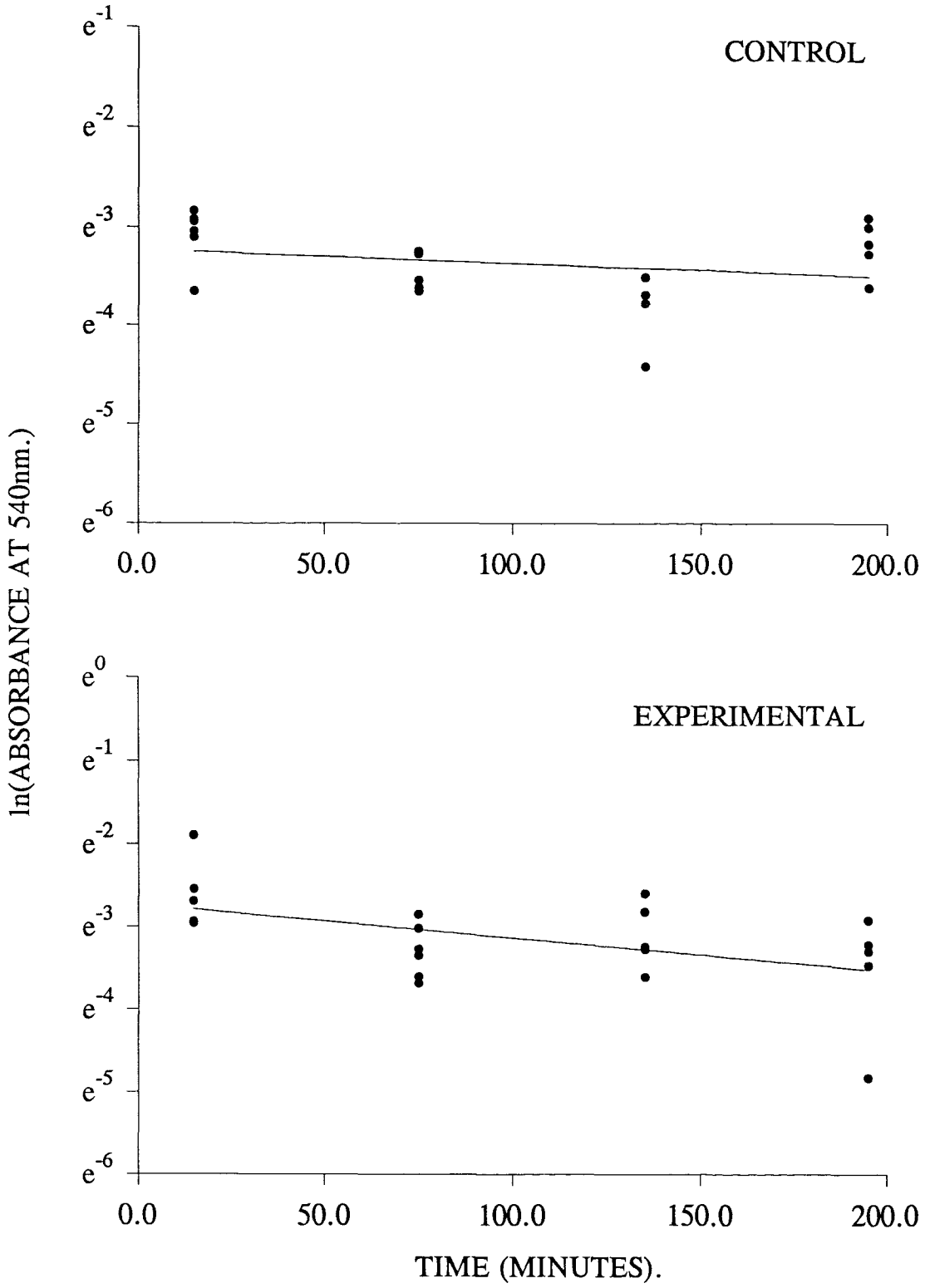

FIGURE 6.07 Comparison of the rates of neutral red release between control and experimental *C. maenas* after the seven day incubation period. Regression statistics have again been summarised in table 6.05

EXPERIMENTAL CONDITION	REGRESSION EQUATION	*P* VALUE	SIGNIFICANT ?
CONTROL + 1 DAY	ln (ABS) = (-0.006 TIME) - 2.59	< 0.001	SIGNIFICANT
EXPERIMENTAL + 1 DAY	ln (ABS) = (-0.006 TIME) - 3.33	< 0.001	SIGNIFICANT
CONTROL + 1 WEEK	ln (ABS) = (-1.42×10^{-3} TIME) - 3.23	0.212	NOT SIGNIFICANT
EXPERIMENTAL + 1 WEEK	ln (ABS) = (-0.004 TIME) - 2.73	0.011	SIGNIFICANT

TABLE 6.05 Regression relations describing the rate of loss of neutral red from haemocytes as a function of incubation period and exposure condition. ABS = Absorbance at 540nm, ln = natural logarithmic function.

6.4 DISCUSSION.

This final investigation has attempted to define for the crab, *Carcinus maenas*, the detrimental effects of a synergistic exposure to the bacterial pathogen, *Listonella anguillarum*, and hydrocarbon pollution (50% WSF Wyche Farm crude oil), using the assays and experimental procedures developed earlier in this research. While synergistic exposures provide a more realistic model of the processes taking place inside organisms in the natural environment they are, by nature of the number of variables operating simultaneously, inherently more complex to interpret. These complexities probably explain, in part, the limited data there are available for comparison. As indicated by Sindermann (1993) and Anderson (1993) examples of the interaction between pathogenic infection and hydrocarbon pollution are "not abundant" in the literature and in most instances have dealt solely with the effects on immune systems overlooking the changes in metabolic status of the exposed organisms. Furthermore in the majority of cases research has been undertaken using molluscan species to act as the model organism which complicates any comparison with the present work. In the shrimp *Penaeus duorarum* the prevalence and intensity of *Baculovirus* infection have been shown to be enhanced by exposure to polychlorinated biphenyls and the insecticide Mirex™, or by the stress associated with over-crowding (Couch & Nimmo, 1974; Couch 1976; Couch & Courtney, 1977) and Nimmo *et al.* (1978) have also demonstrated immunosuppression in cadmium exposed shrimp. The current investigation therefore represents the first comprehensive study of the effects of a combined exposure to oil pollution and bacterial infection on the metabolic and immune status of *Carcinus maenas*.

One of the most obvious results of this synergistic incubation was the death of one of the experimental crabs during the phenoloxidase study. It is true that this mortality could have been due to some unrelated and undefined process such as a viral infection (viral infections of *C. maenas* have been reported by Bang (1971) and Johnson (1983) and for other crab species by Sindermann & Lightner (1988)) however, it is interesting to speculate as to why the death should have occurred in

this incubation alone. It is feasible that the combination of a sub-lethal exposure to hydrocarbon pollution and a sub-lethal pathogenic infection could have proved sufficient to induce mortality in this one, perhaps slightly more susceptible, individual. Certainly Khan (1991) has demonstrated increased mortalities in winter flounder exposed to both hydrocarbon pollution and parasitic infection compared to exposure to either stressor in isolation. With this in mind it would perhaps have been more informative to incubate the crabs for longer to determine whether or not any mortality occurred in other individuals and this certainly would be a consideration in the planning of future studies.

A second general observation which can be made of the data is that the total sugar concentrations were generally higher (thirty to sixty mg. per one hundred ml.) than those measured during the hydrocarbon work (see section 5.3.1). Possible causes of this discrepancy were suggested in chapter five (see section 5.4) and it is not proposed to repeat them here. These differences do however emphasise the problems associated with comparing the data of studies which are of slightly different design and which have been carried out on different occasions and in some instances by different research groups.

Following the bacterial infection described in chapter four very little obvious change was noted in the carbohydrate composition of the infected animals. Slight elevations in the haemolymph sugar concentration were explained in terms of handling stress (Telford, 1974b). As a consequence of the hydrocarbon exposure described in chapter five a reduction in metabolic activity was recorded, which was evidenced by increased sugar levels in the haemolymph and reduced titres of the metabolic end product hydrogen peroxide. This reduced activity associated with oil exposure was superimposed onto a general decline seen as a function of continued starvation. Following the synergistic exposure to both stressors used in this chapter there was an increase in the concentration of haemolymph total sugar which was slightly less in the case of the experimental animals; this was accompanied by a decrease in the concentration of hydrogen peroxide seen for both the control and experimental crabs. It can be concluded therefore that, in a similar manner to the data presented in chapter five, there was

a reduction in the metabolic activity of both the control and experimental animals as a function of continued starvation. This reduction in metabolic activity was slightly less in the case of the experimental crabs probably due to the energetic demand of containing a bacterial infection.

In chapters three and four the potential roles of trehalose in decapod crustaceans were discussed and a number were discounted including the role of stabilising cell membranes. One possible function of trehalose which was tentatively supported by the data was as a "sponge" for roaming superoxide radicals. Following the hydrocarbon exposure of chapter five no correlation between hydrogen peroxide and trehalose was observed although the potential absence of superoxide elicitors was noted. Similarly, in this synergistic study there was no apparent correlation between the concentrations of haemolymph hydrogen peroxide and the glucose dimer trehalose even in spite of the presence of an *in vivo* bacterial infection. It must be remembered however, that the presence of trehalose in the haemolymph of *Carcinus maenas* is a complex function of a number of different processes including the catabolism of glycogen to glucose (gluconeogenesis) as well as the reverse formation of glycogen reserves. Moreover it is probable that such processes are tissue specific as suggested by Hohnke & Scheer (1970) and discussed in chapter three. With these implications in mind it is very difficult to be clear as to the significance of trehalose within the circulation. Two avenues for further research into this problem would be:

1) The comprehensive radiolabelling of a number of different haemolymph sugars in an attempt to visualise their destinations and various roles (similar to the techniques employed by Hill *et al.* (1991) to monitor the metabolic changes associated with environmental anoxia in *C. maenas*).

2) An *in vitro* investigation of the potential of trehalose to absorb superoxide ions and produce hydrogen peroxide to determine from stoichiometric principles whether or not such reactions could be feasible *in vivo*.

In the interim it can be concluded that, unlike the observations of Telford (1968) describing an absence of the dimer in the haemolymph following handling stress in *Homarus americanus*, trehalose is always present in the haemolymph of *Carcinus maenas*.

One overriding difference between this synergistic study and the previous data discussed in this thesis is the extremely large contribution (50-70%) made by the "other sugars" component to the total carbohydrate pool. The composition of this pool of "other sugars" has been previously discussed (see chapter four and Johnstone & Spencer-Davies, 1972) and conceivably a proportion of this group is due to maltose, a product of the catabolism of glycogen; glucosamine, the N acetyl polymer of which forms chitin; and galactose which for the experimental crabs could possibly have derived from bacterial cell walls. Questions must be raised as to why this component is so large in both the control and experimental animals in this study and at the same time why the glucose contribution is so small. The small glucose component could represent an adaptation to the aquarium environment in which the animals were maintained prior to the experimental incubations. Unlike the previous studies described in earlier chapters the crabs used in this synergistic exposure had been held in the aquarium for an extended period of time (between four and six weeks) and during this time they were fed on alternate days with an excess of coley (*Pollachius virens*). This diet may have resulted in a physiological adaptation by the crabs to build up polysaccharide reserves. This suggestion is entirely speculative and would need verification by long term studies on the effects of diet on the carbohydrate composition of aquarium maintained animals.

The previous investigations of haemocytic responses to bacterial infection and hydrocarbon exposure have both demonstrated a rapid decline in the total haemocyte count (THC) for exposed crabs. Similarly in this study reductions in haemocyte number are seen for both the control and experimental crabs. The decline in cell population of the control crabs is probably a function of the continued starvation as has been discussed in chapter five. Superimposed on this effect in the experimental animals is a response to the oil exposure and bacterial

infection. In the previous chapters the idea of cell lysis was discounted due to the absence of any cell debris seen in the haemocytometer counting chamber and the equivocal neutral red retention data. Once again in this synergistic exposure, no cell debris was observed in any of the haemolymph samples collected from the experimental crabs and on this occasion the neutral red retention assay indicated that the haemocyte stability actually increased during the week incubation. It is probable that the reduction in the number of circulating cells was due to their sequestration within the gill filaments, both as nodules encompassing bacteria and as a function of the oil exposure discussed in chapter five. Figure 6.04 indicates that proportionally there were slightly more phagocytic hyalinocytes removed from the circulation of the experimental crabs, an observation echoed by the data recorded following bacterial infection (see chapter four). It can therefore be concluded that the phagocytic hyalinocytes are preferentially retained within the gill tissue following a synergistic exposure to oil pollution and bacterial infection. Smith & Ratcliffe (1978) have previously demonstrated the role of the phagocytic cells in *Carcinus maenas* following bacterial infection. These authors have demonstrated the ability of *C. maenas* to rapidly clear bacteria from its circulation by virtue of the phagocytic nature of its hyaline cells.

One anomalous observation of the present study was the initial rise in the THC of the exposed crabs twenty-four hours after the incubation began. In both the bacterial and hydrocarbon studies total haemocyte counts were rapidly reduced in the experimental crabs - a reduction which was maintained throughout the incubation. The observed data in this study remain difficult to explain, however it must be remembered that the difference between the control and experimental crabs was not significant at this time and the discrepancy may merely emphasise the high degree of individual variation witnessed repeatedly throughout this research.

The phenoloxidase enzyme activity data collected during this synergistic study demonstrated that there was no difference between the control and experimental crabs throughout the incubation. Following a bacterial infection (chapter four) there was an initial and predictable decrease in the phenoloxidase

activity of the infected crabs followed by a slight increase by the end of the week incubation which was suggestive of some form of immunostimulation. As discussed in chapter five hydrocarbon exposure seemed to have no inhibitory effect on the phenoloxidase activity of experimental crabs. However, when assayed together it would seem that the phenoloxidase responses to bacterial challenge were being inhibited in some way. It is conceivable that hydrocarbon exposure, whilst not directly inhibiting the activity of the phenoloxidase enzyme may have acted as an immunosuppressant in some other part of the immune system which in turn influenced the activity of the phenoloxidase enzyme. Whilst the idea of a phenoloxidase cascade has now been discredited as inaccurate (Söderhäll, 1994; pers. comm.) there are many different stages to the phenoloxidase system such as the proteolytic cleavage by a serine proteinase of a five kilodalton subunit from the phenoloxidase precursor activating the zymogen (Ashida & Yoshida, 1988). The hydrocarbon exposure may influence one of these initial stages, only becoming obvious when the system attempts to respond to pathogenic challenge. One potential site of inhibition is the β 1,3-glucan receptor on the cell membranes of the granulocytes (see review by Söderhäll, 1992). These receptors bind to bacterial glucans and initiate the release of inactive zymogen into the plasma where upon it is activated. This inhibition could be achieved by two mechanisms, the first of which is analogous to the competitive inhibition of enzymes. It is possible that small organic molecules may have directly competed with the β 1,3-glucans for binding sites on the surface of the granulocytes. Inhibition may have also been achieved through a reduction in the number of binding sites on the surface of the granulocytes, analogous to non-competitive inhibition. Sami *et al.* (1990) demonstrated a reduction in the number of concavalin A binding sites on oyster (*Crassostrea virginica*) haemocytes following hydrocarbon exposure and it is conceivable that a similar mechanism was operating in this instance. Inhibition of the phenoloxidase system at this binding site, via either mechanism, by low molecular weight organics would not have "shown up" during the hydrocarbon study (chapter five) as it would have had no affect on the cellular prophenoloxidase distribution. However, inhibition at this site would have prevented the release of prophenoloxidase from these cells in response to bacterial

stimulation and in so doing would have prevented the changes in enzyme activity seen in chapter four. It is clear that the immunosuppressive effects of xenobiotics on *C. maenas* may be complex and subtle and certainly the role of hydrocarbon exposure on the small scale detail of the phenoloxidase system warrants further research.

The neutral red retention assay proved to be more conclusive in this synergistic study than in the last chapter. A day after the incubation had started both the control and experimental haemocytes had identical rates of dye release suggesting that the cells were equally as labile. Over the week incubation the integrity of those cells that remained in circulation increased, more so in the case of the control animals than in the experimental crabs. This suggests that there was some stress associated with the incubation procedure which was adapted to during the week incubation. The concurrent pathogen challenge and exposure to hydrocarbon pollution in the experimental animals reduced the ability of the *C. maenas* to maintain an optimal internal environment and subsequently the cellular integrity of the experimental animals did not make as complete a recovery as did the control animals.

It is clear from the above discussion that the combination of two separate stressful conditions produces complex changes in the metabolism and immunology of *C. maenas*, not all of which can be predicted or even explained with the current level of knowledge. The data presented in this chapter demonstrate the real problem associated with the application of theoretically derived indices which have been developed and tested in a controlled laboratory environment to natural situations in which more than one variable operate simultaneously. The most predictable change observed in *C. maenas* during this synergistic exposure was the decrease in the total haemocyte count and once again it would seem that this index has the greatest potential for monitoring stress associated changes in the internal condition of the shore crab. To attempt to explain the changes in metabolic status of *C. maenas* following this exposure requires an extensive investigation of the *fluxes* associated with the haemolymph sugars rather than trying to make

inferences from static "snapshots" of the internal environment. The role of trehalose in particular remains enigmatic although its involvement in membrane stability in adult decapod crustaceans is questionable. The idea that trehalose may sequester and neutralise roaming superoxide radicals whilst plausible is, at best, tentative speculation. From *in vitro* studies (Bell & Smith, 1993) the idea of a respiratory burst response in decapod crustaceans has only recently been suggested and this clearly needs further validation. At the same time the mechanics of the phenoloxidase system require further elucidation although the degree of individual variability in this measure questions its usefulness as an indicator of marine crustacean immunocompetence.

The experiment described in this chapter uses the general protocols adopted from the previous investigations and, as such, is constrained by the same limitations as have been previously described. For example it must be remembered that the oil exposure method used in this study produced a very pulsed hydrocarbon load which is perhaps not realistic of the natural environment. During this incubation only one crab died and no lethal infection (evidenced by milky white haemolymph) was seen to take hold in the remaining crabs. More conclusive data may have been generated had higher doses of bacteria and oil been administered. This would have however, increased the incidents of mortality amongst the experimental crabs conflicting with one of the key tenets of this research, specifically to investigate sub-lethal responses.

CHAPTER SEVEN

GENERAL CONCLUSIONS AND PROPOSALS FOR FUTURE RESEARCH

CHAPTER SEVEN

GENERAL CONCLUSIONS AND PROPOSALS FOR FUTURE RESEARCH

7.1 GENERAL SUMMARY AND SELECTION OF SUITABLE INDICES.

The aims of the research described in the preceding chapters were to assess the potential of *Carcinus maenas* to act as a biomarker of water quality and to comprehensively investigate the metabolism and immunology of this well-documented species. In the introductory chapter the use of mobile crustaceans as potential biomarkers was justified in spite of the fact that they contravene the second prerequisite indicated by Phillips (1980). It can be argued that in modern aquaculture systems crustaceans are often held at high densities in confined spaces, an example being crab shedding tanks. In containment systems such as these the ability of the individuals to avoid stressful situations is severely impeded. At the same time crustacean species represent a different set of immunological and metabolic systems which have their own unique responses to imposed stresses. For example, crustaceans do not have immunoglobulins within their immune systems as is the case for vertebrate species and, unlike molluscs, they do not have the ability to preferentially produce succinate when faced with anoxic conditions (de Zwaan 1977 & 1983, reviewed by Storey & Storey, 1990).

In this study a number of different indices were developed to indicate the metabolic status and immunocompetence of the sampled crabs. Measurements of haemolymph total sugar, glucose, trehalose and hydrogen peroxide were made to provide information on the metabolic status of the experimental individuals and immunocompetence was assessed by measuring total and differential haemocyte populations and by determining the activity of the phenoloxidase enzyme. Of course the division of indices into "metabolic" and "immunological" is artificial and a certain degree of overlap has been demonstrated in this thesis but

nevertheless the classification remains useful as a basis for ordering discussion.

Before any index of organism condition can be implemented for use in biomonitoring programmes the baseline variability associated with "natural" environmental perturbation first needs to be assessed. To this end the first two experimental chapters in this thesis have described investigations into the variation associated with regular tidal cycles using a computer controlled tidal tank (chapter two) and also the changes associated with the seasonal cycle (chapter three). The duration of these two baseline studies also provided a period of time in which to develop all of the indices that were to be used in the research.

Tidally associated variability in the condition of *C. maenas* was assessed in a controlled laboratory environment in which all the other impinging variables were regulated. Sinusoidal cycles of tidal height were produced using a computer controlled tidal tank with an effective tidal excursion of approximately one metre. The results of this study indicated that there were predictable changes in the metabolism and immunocompetence of the shore crab associated with the change in water depth. For continuously submerged individuals (analogous to crabs with a sublittoral distribution but within the zone of tidal forcing) high water was demonstrated as a period of increased locomotor activity which was reflected by high rates of oxidative metabolism. This was seen in terms of a low haemolymph total sugar concentration and a high hydrogen peroxide concentration at high water with the reverse during low water. Indeed, a significant negative correlation between haemolymph total sugar and hydrogen peroxide was demonstrated for the first time in this study. Furthermore these periods of increased locomotor activity coincided with an enhanced level of immunocompetence. High water was recorded as a time of elevated haemocyte populations and reduced prophenoloxidase stores represented by the measure "phenoloxidase enzyme activity." The idea of enhanced immunocompetence during periods of high activity has been suggested before for *C. maenas* by Truscott & White (1990) and also for *Mercenaria mercenaria* by Hawkins *et al.* (1993) but this is the first occasion on which circatidal rhythmicity in the activity of the phenoloxidase enzyme has been demonstrated (Hauton *et al.*, 1995).

Entrainment to a tidal cycle that included two hours of aerial exposure around low water (analogous to the situation faced by crabs living on shorelines) produced a complete reversal of the trends of immunocompetence and metabolic status. The cellular store of prophenoloxidase was seen to be at a minimum during aerial exposure which was explained in terms of a metabolic rate depression during periods of haemolymph oxygen starvation. The low sugar, high hydrogen peroxide and elevated haemocyte population seen during emersion initially appeared to be counter-intuitive in light of the data recorded for the continuously submerged crabs. An hypothesis was constructed to explain these data in terms of a reduction in cardiac activity during periods of air exposure which would have allowed the haemolymph to collect in peripheral sinuses potentially giving rise to the data obtained. This hypothesis was tested by measuring changes in cardiac output (both in terms of the heart rate and the mean amplitude of the cardiac contraction) associated with entrainment condition and tidal state. Whilst the data collected from this subsequent investigation were inconclusive the idea of a differential haemolymph distribution during periods of aerial exposure (Airriess & McMahon, 1994) could not be discounted. It is evident that the metabolic and immunological changes associated with repeated aerial exposure require further investigation as do the subtle changes in cardiac activity and haemolymph distribution.

The data presented in chapter three indicated that there were no predictable seasonal cycles in the measured indices of organism condition possibly because of the number of environmental variables operating simultaneously. Further to this the data collected proved to be extremely variable emphasising the high degree of individual variation seen repeatedly throughout this research. In spite of these two problems the data collected did indicate some interesting correlations between the environmental conditions and the indices of organism condition. A significant positive correlation between total haemocyte counts and salinity was observed and the potential role of haemocytes augmenting the free amino acid pool and regulating osmotic pressure was suggested. Phenoloxidase enzyme activity, indicative of the cellular store of the zymogen prophenoloxidase, was seen to increase with increasing bacterial load in the water column. This study represented

the first recorded incidence of any such correlation in marine crustaceans and the idea of immunostimulation of the phenoloxidase system was discussed. The metabolic data were extremely variable and no obvious correlations were demonstrated. One important observation was the lack of any correlation between haemolymph total sugar and hydrogen peroxide which led to the suggestion that the presence of hydrogen peroxide within the haemolymph was not solely a function of routine metabolism and that a proportion of the peroxide measured was derived from superoxide production. One of the potential roles of haemolymph trehalose is to act as a "sponge" for roaming superoxide radicals and the positive correlation between haemolymph trehalose and hydrogen peroxide supported the idea of non-specific immunostimulation. In conclusion, it was clear from this study of seasonally associated baseline variability that, whilst the changes in the measured indices may not have been cyclical and predictable, correlations between indices and environmental parameters were evident and would need to be taken into consideration in any biomonitoring programme.

Once the baselines of variability had been defined with these first two studies the responses of *C. maenas* to a variety of artificially imposed stresses could be monitored and the subsequent chapter (chapter four) dealt with the first of these artificially induced stresses, that of a pathogenic infection. This study proved novel because a live inoculation of the Gram negative bacteria *Listonella anguillarum* was used in favour of the denatured inoculations of previous workers.

The data presented in chapter four indicated that a sub-lethal live infection had very little influence on the metabolic status of *C. maenas* however, subtle changes in the composition of the carbohydrate pool were evident. The most obvious change in the haemolymph carbohydrate was an increase in the percentage contribution made by the glucose dimer trehalose in the infected crabs during the seven day incubation. This increase significantly correlated with an increase in the concentration of hydrogen peroxide and again led to the suggestion of superoxide production following stimulation by pathogens *in vivo* and the role of trehalose in soaking up unused radicals. More conclusive effects of a bacterial infection were seen in relation to the haemocyte population with significant decreases in the number of circulating haemocytes being recorded following inoculation with

Listonella anguillarum. The haemocytes were removed to the gill filaments where they sequestered bacteria producing localised inflammatory responses and causing extensive tissue damage. Phenoloxidase enzyme activity was again variable although following pathogenic infection a reduction in the cellular store of zymogen was apparent which recovered to a level greater than the control animals after one week's incubation. The phenoloxidase enzyme activity data suggested an immunostimulation response following a sub-lethal pathogenic infection and was the first time any such mechanism has been recorded in *C. maenas*.

The second artificial stress imposed on specimens of *Carcinus maenas* in the laboratory, that of hydrocarbon exposure, was discussed in chapter five. Very little change in the metabolic status of the shore crab was seen after one week's incubation in a fifty percent dilution of the water soluble fraction of Wych Farm crude oil. A reduction in the metabolic activity was demonstrated in both the control and experimental crabs during the week which was explained by the absence of any dietary intake. The reduction in metabolism was slightly accelerated in the hydrocarbon exposed crabs which led to the suggestion of biochemical suppression by organic compounds within the crude oil fraction. Oil exposure was seen to cause a significant reduction in the circulating haemocyte population during the week incubation but apparently had little influence on the highly variable activity of the phenoloxidase enzyme.

The final study in this thesis described the effects of a synergistic exposure to hydrocarbon pollution and bacterial infection on the immunocompetence and metabolism of *C. maenas*. Whilst this represented a more realistic situation it was, at the same time, inherently more complex and some of the data collected remain difficult to explain. Once again little change was demonstrated in the metabolic status of crabs synergistically exposed. A reduction in metabolic activity was seen in both the control and experimental crabs similar to that described in chapter five. The rate of reduction in activity was slower in the experimental crabs, which contradicted the observations recorded following hydrocarbon exposure alone, and which was explained in terms of the metabolic requirements of containing the bacterial infection in the synergistic exposure. As with the earlier single exposures a reduction in the haemocyte population was demonstrated in the experimental

crabs in the synergistic exposure and this was again concluded to be the most predictable observation recorded.

Perhaps the most interesting observation of this final study was that the phenoloxidase enzyme system did not appear to respond to the presence of bacteria as seen in chapter four. It was concluded that whilst hydrocarbon exposure alone appeared to have no influence on the cellular store of the zymogen prophenoloxidase it prevented this store being released in response to pathogenic infection possibly by inhibition of the β 1,3-glucan binding sites. This was the first occasion on which the potential immunosuppression of the phenoloxidase system has been suggested for marine crustaceans.

In both of the last two experimental chapters data were presented relating to the retention of the dye neutral red by haemocytes of *C. maenas*. This *in vitro* assay was modified to measure the stability of the haemocytes and to clarify the roles of the disaccharide sugar trehalose. From the data presented in chapter five and chapter six it was apparent that there was little change in the membrane stability of the circulating haemocytes and this led to the suggestion that the fluctuations in haemolymph trehalose indicated in this research were not a function of maintaining cellular stability. It was concluded that the presence of trehalose within the haemolymph of crabs was due to a combination of the metabolism of glycogen to glucose and also possibly as a self protection mechanism against the non specific activity of superoxide radicals released in response to pathogenic stimulation.

From the data presented in this thesis it is evident that the metabolism of *Carcinus maenas* is very resistant to the stresses imposed by pathogenic infection and hydrocarbon exposure. The metabolic trends observed when the data were recorded were invariably masked by a high level of individual variation and there was usually little consistent correlation with the magnitude or the nature of the stress imposed. As a consequence of the resilience exhibited by the indices of metabolic status their consideration as potential biomarkers of organism condition is unjustified.

At the same time the phenoloxidase enzyme data presented in this thesis

were extremely variable and its implementation as an index of immunocompetence would also prove problematic. Furthermore the activation mechanism and the potential roles of the phenoloxidase enzyme within the Crustacea have yet to be fully determined and hence it is difficult to predict the consequences of changes in the activity of the enzyme on the immunocompetence of the species concerned. Consideration of phenoloxidase enzyme activity as an index of organism condition is therefore also restricted.

Of all of those tested the only index to produce relatively consistent and predictable data was that of haemocyte counts. Significant decreases in haemocyte population were seen following pathogenic infection and exposure to organic pollution and a similar response was seen when the two stressors were combined. The decreases in cell population could always be explained and the consequences of such depletions were predictable. It would therefore seem that measures of both differential and total haemocyte population offer the most potential for monitoring the condition of estuarine crustaceans.

7.2 *Carcinus maenas* - A POTENTIAL BIOMARKER OF WATER QUALITY?

The potential of *Carcinus maenas* to act as a biomarker of water quality must now be considered. *C. maenas* is a useful species to study as it is a dominant member of coastal and estuarine benthic communities and can be collected in sufficient numbers to allow meaningful experimental comparisons. Further to this *C. maenas* is a "hardy" species readily adapting to transfer from natural to artificial environments and is robust enough to allow experimental manipulation. Indeed Bayne *et al.* (1985) have argued that estuarine eurytopic organisms, such as *C. maenas,* have the greatest potential as biomarking organisms as they are naturally resistant to environmental perturbation. In the introductory chapter this view was questioned however, and a case was presented for the use of less resistant, more sensitive organisms as biomarkers. In this study *C. maenas* has been presented with a virulent species of bacteria (Chisholm, 1993) and the water soluble fraction of a light crude oil which is dominated by toxic hydrocarbons and

yet the data collected have in some instances proved inconclusive or highly variable. From these data it is apparent that the inherent resilience exhibited by *C. maenas* may make the species too insensitive to monitor *sub-lethal* declines in water quality. For example, the metabolic data presented following hydrocarbon exposure were equivocal and had a more stenotopic organism been selected more definitive trends may have been observed. The principle of using crustaceans as biomarking organisms does however, remain valid and future research should perhaps consider the use of less versatile species such as the swimming crab (*Liocarcinus depurator*) or even the edible crab (*Cancer pagurus*) which are both less tolerant of salinity and temperature fluctuations and are entirely sublittoral in their distributions.

7.3 FUTURE RESEARCH CONSIDERATIONS.

Several potential avenues of further research stem from the current work and these have been summarised below:

1) An investigation of *Liocarcinus depurator* and *Cancer pagurus* to determine if the responses to stress demonstrated in *Carcinus maenas* are applicable to other crustacean species and to assess whether or not these less resilient species produce stronger or more predictable responses. These two sublittoral species which are less tolerant of environmental perturbation would be expected to exhibit similar responses to *C. maenas* but be more sensitive to the stresses imposed and therefore produce more consistent data.

2) Investigations into the variation associated with sex, age/moult stage, and colour morph in *C. maenas*. These considerations were specifically avoided in the current research but nevertheless would require investigation. However, with the individual variation seen for adult males of greater than fifty-five millimetres carapace width, the data collected for these other divisions may also prove vague and difficult to analyse.

3) An investigation into the *fluxes* of carbohydrates in *C. maenas* under the experimental conditions used in the present study. The radio- or fluorescent labelling of sugar compounds (Hill *et al.*, 1991) would clarify the static "snapshot" results of the current research. The fate of labelled carbohydrates, such as ^{14}C labelled glucose, trehalose or glycogen, introduced into *C. maenas* could be monitored to determine the fluxes taking place during pathogenic infection or hydrocarbon exposure. For example, if labelled glycogen was injected into *C. maenas* and appeared as labelled trehalose and glucose within the haemolymph it could be concluded that polysaccharide energy reserves were being broken down to produce substrates for oxidative metabolism during periods of reduced dietary intake.

4) Labelling of those free amino acids within the haemocytes to determine the osmoregulatory potential of haemocytes within the gills following exposure to high salinities (chapter three) or hydrocarbon pollution (chapter five). If, following exposure to either of these stressors, labelled amino acids were isolated from epithelial cells within the gill filaments it would support the theory that haemocytes have the potential to regulate the osmotic potential within the haemolymph and gills of *C. maenas.*

5) Stoichiometric *in vitro* study of the potential of the glucose dimer trehalose to absorb superoxide ions and produce hydrogen peroxide to determine whether or not this process is feasible *in vivo*. The role of trehalose in absorbing superoxide radicals has only been conclusively demonstrated in the mycelia of *Cunninghamella japonica* (Tereshina *et al.*, 1991) and the stoichiometry and kinetics of the reactions involved may not be reproducible within the haemolymph of *C. maenas*.

6) A more extensive investigation of the phenoloxidase system to determine whether immunostimulation following sub-lethal bacterial infection can be repeated for other bacterial species. It is conceivable that

immunostimulation of the phenoloxidase system may be dependent on the species of pathogen used and less virulent species such as *Psychrobacter immobilis* (Chisholm, 1993) may prove insufficient to stimulate the phenoloxidase system. Further to this an investigation of the sites of inhibition of the phenoloxidase system by hydrocarbon pollutants.

7) Development of a suitable neutralising assay to measure plasma phenoloxidase and provide information on the *in vivo* active fraction (Hauton *et al.*, 1995). From the current theories relating to the phenoloxidase system (discussed in chapter one) it is expected that there would be a consistent negative correlation between the amount of enzyme stored within the haemocytes and the amount that is active within the plasma *in vivo*.

8) A more extensive study of cardiac physiology to examine the possibility of a differential haemolymph distribution during periods of aerial exposure. The work of Airriess & McMahon (1994) used totally restrained and partially dissected crabs which proved too restrictive for adoption in the present work - but some means of recording differential haemolymph flow on unrestrained crabs needs to be made. As suggested in chapter two, whilst no changes in the heart rate or the amplitude of contraction were recorded in the present study the flow of haemolymph may be redirected to preferentially feed the sternal artery during periods of aerial exposure producing the observed haemocyte and metabolic data in the tidally exposed crabs.

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APPENDICES

APPENDIX A

FORMULATION OF REAGENTS

APPENDIX A.1 HAEMOLYMPH SUGAR DETERMINATION (Roe, 1955).

Anthrone Reagent:

A solution containing 0.05% anthrone, 1% thiourea, and 66% by volume concentrated sulphuric acid was used. For each 500ml. used 170ml. of deionised water was placed in a pyrex beaker and 330ml. of concentrated sulphuric acid (sp. gr. 1.84) was added cautiously. To this was added 250mg. of recrystallised anthrone and 5g. of thiourea. The solution was then gently heated to 85°C before being allowed to cool. The solution was made up fresh every two weeks and was stored in a refrigerator.

Deproteinising Reagent:

A 5% (v/v) solution of trichloroacetic acid was made using deionised water. The solution was stored in a refrigerator and was stable indefinitely.

Glucose Standard:

A stock solution of 1mg. $ml.^{-1}$ was produced by dissolving 100mg. of oven dried glucose (70°C for 12 hours) in 100ml. of saturated benzoic acid water in a volumetric flask. From this working standards were made by diluting 10ml. of the stock in a 100ml. volumetric flask with saturated benzoic acid, giving a final concentration of 0.1mg. $ml.^{-1}$ (10mg. $100ml.^{-1}$). In the first instance a range of standards were produced to make a standard curve (figure A1.01) from which agreement with the Lambert & Beer Law was confirmed.

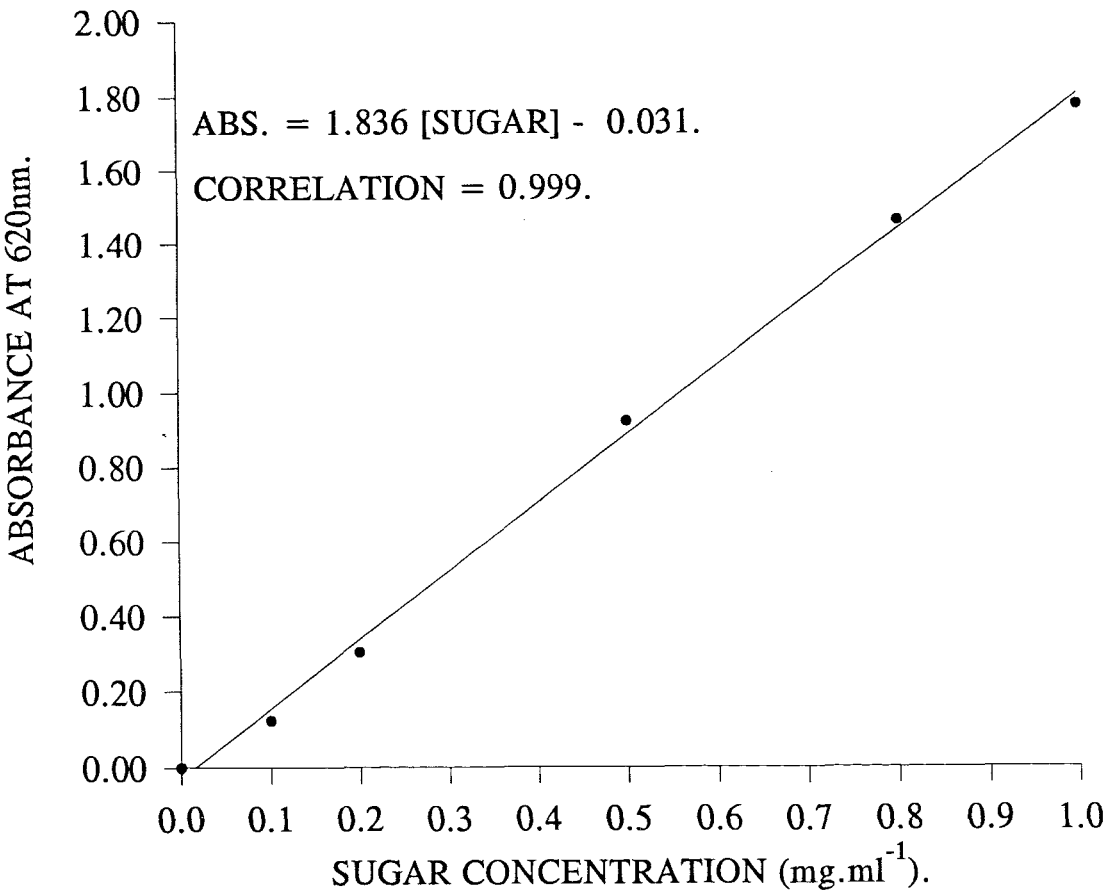

FIGURE A1.01 Standard curve for sugar determination confirming a linear relationship between sugar concentration and absorbance at a wavelength of 620nm. for concentrations in the range 0.0 to 100.0mg. per one hundred ml.

APPENDIX A.2 HAEMOLYMPH GLUCOSE ASSAY (Bayne *et al.*, 1985).

ATP Buffer:

TEA stock, 0.5M., pH = 7.6: 93.0g. of triethanolamine hydrochloride buffer (TEA) were added to 400ml. of deionised water, the pH was adjusted to 7.6 using a solution of 1M. sodium hydroxide and then the solution was made up to one litre with deionised water. This stock solution was stored at 4°C.

20mM. Nicotinamide adenine dinucleotide phosphate (oxidised) (NADP+): A solution of 20mM. NADP+ was made using deionised water. 0.5ml. aliquots of this stock solution were stored frozen ready for use.

Stock ATP Buffer: 3ml. of 0.5M. magnesium chloride hexahydrate were added to 285ml. of diluted (x10) TEA stock solution. The stock buffer was made up in advance and stored in an amber bottle at 4°C.

Working ATP Buffer: Immediately before use a 0.5ml. aliquot of 20mM. NADP+ was thawed and added to 48ml. of stock ATP buffer.

Enzymes:

Glucose-6-phosphate dehydrogenase (G-6-PDH): A solution of enzyme containing 0.6mg. protein ml^{-1} in 3.2M. ammonium sulphate was made and stored at 4°C.

Hexokinase (HK): A solution of enzyme containing 2.0mg. protein ml^{-1} in 3.2M. ammonium sulphate was made and stored at 4°C.

Glucose standard:

5ml. aliquots of 50mg. glucose per ml. in deionised water were made using predried (70°C, 12 hours) pure glucose. All of the aliquots were stored frozen until immediately prior to analysis.

APPENDIX A.3 HAEMOLYMPH HYDROGEN PEROXIDE DETERMINATION (Meiattini, 1984).

Peroxide Reagent:

A 100ml. solution containing:

2670mg. Disodium hydrogen orthophosphate

1726mg. Sodium dihydrogen orthophosphate

100mg. Sodium azide

100μl. Triton X-100™

4mg. Peroxidase Enzyme

10mg. 4-aminophenazone

400mg. Chromotropic acid (hydrated sodium salt)

- was made using deionised water and a volumetric flask. This solution was stored in a stoppered brown glass bottle at 4°C and replaced each month. For this reaction the 4-aminophenazone/chromotropic acid complex acted as a hydrogen donor whilst the azide was added to prevent bacterial contamination of the samples and to inhibit any catalase which may have been present and the Triton X-100™ helped to solubilise any lipid fractions.

Deproteinising Reagent:

A solution of 1M. perchloric acid was made by diluting 8.6ml. (70% w/w, sp gr 1.67) perchloric acid to 100ml. with deionised water.

Neutralising Solution:

A 6M. solution of sodium hydroxide was made by slowly dissolving 24gm. of sodium hydroxide in about 70ml. deionised water before making up to 100ml. This solution was stored in a stoppered plastic bottle.

Hydrogen Peroxide Standard:

A stock solution of standard was made by diluting 1.0ml. hydrogen peroxide (ca. 35% w/w) in a 250ml. volumetric with deionised water. The

solution was calibrated prior to each use by diluting 20ml. of stock with 30ml. of water and adding 5ml. of a solution of 0.5M. sulphuric acid. The mixture was titrated against 0.02M. potassium permanganate to a permanent pink colour. According to the results of the titration (1.0ml. of $KMnO_4$ = 1.7mg. hydrogen peroxide) a working standard of 20mg.l^{-1} was made by diluting the appropriate volume of stock with deionised water. As for the sugar determination in the first instance a range of standards was produced to give a calibration curve (figure A3.01) with which to confirm agreement with the Lambert & Beer Law.

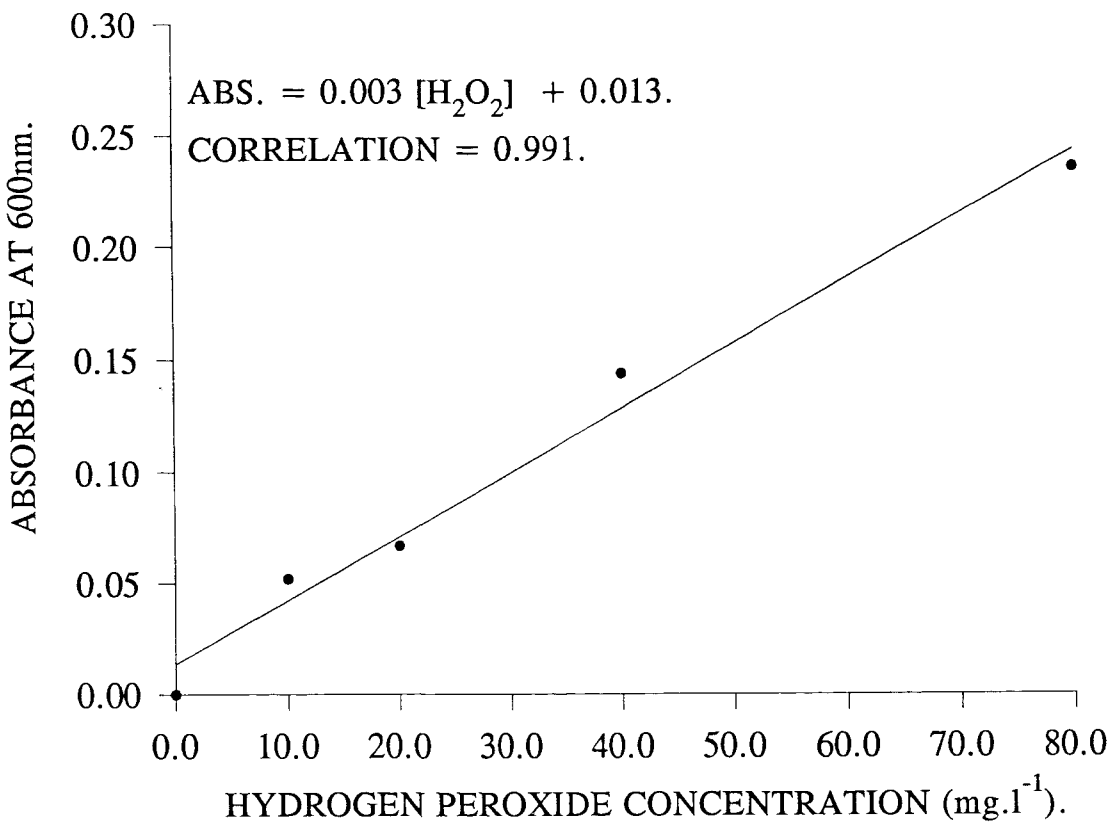

FIGURE A3.01 Standard curve for hydrogen peroxide determination confirming a linear relationship between hydrogen peroxide concentration and absorbance at a wavelength of 600nm. for concentrations of hydrogen peroxide in the range 0.0 to 80.0mg.l^{-1}.

APPENDIX A.4 PHENOLOXIDASE ENZYME DETERMINATION.

Marine Anticoagulant pH = 4.6 (Söderhäll & Smith, 1983):

A solution containing:

0.45M. Sodium Chloride

0.1M. Glucose

30mM. Trisodium Citrate

26mM. Citric Acid

10mM. EDTA

- was made using"Milli-Q™" water and acid cleaned and sterile glassware. The solution was stored at 4°C.

Sodium Cacodylate Buffer pH = 7.0 (Jackson *et al.*, 1993):

A solution containing:

10mM. Sodium Cacodylate

150mM. Sodium Chloride

10mM. Hydrated Calcium Chloride

- was made using "Milli-Q™" water and acid cleaned and sterile glassware and stored at 4°C.

Sodium Cacodylate Citrate Buffer pH = 7.0 (Jackson *et al.*, 1993):

A solution containing:

10mM. Sodium Cacodylate

100mM. Trisodium Citrate

0.45M. Sodium Chloride

- was made using "Milli-Q™" water and stored at 4°C.

Phenoloxidase Activator:

A solution of 0.1% trypsin (0.5 Anson units per gramme from beef pancreas) in cacodylate buffer was used as the zymogen activator. As before this solution was stored at 4°C until use.

Phenoloxidase Assay Substrate:

A 4g.l^{-1} solution of L-dihdroxyphenylalanine (L-dopa) in "Milli-Q™" water was made up as required. The substrate stability was very poor and as a result 0.052g. were dissolved in 13ml. "Milli-Q™" water immediately prior to use.

Protein Determination, Biuret Reagent:

1.5g. hydrated copper sulphate and 6.0g. of hydrated sodium potassium tartrate were dissolved in 500ml. deionised water. 300ml. of a solution of 0.1% sodium hydroxide were slowly added with continuous stirring before the final solution was made up to one litre. The solution was stored in a refrigerator and was stable indefinitely.

Protein Determination, Protein Standard:

A 10mg.ml^{-1} bovine serum albumen (fraction V) standard was made using deionised water and stored in a refrigerator. As before a range of standards were initially produced to ensure a linear relationship existed between protein concentration and optical density (see figure A4.01).

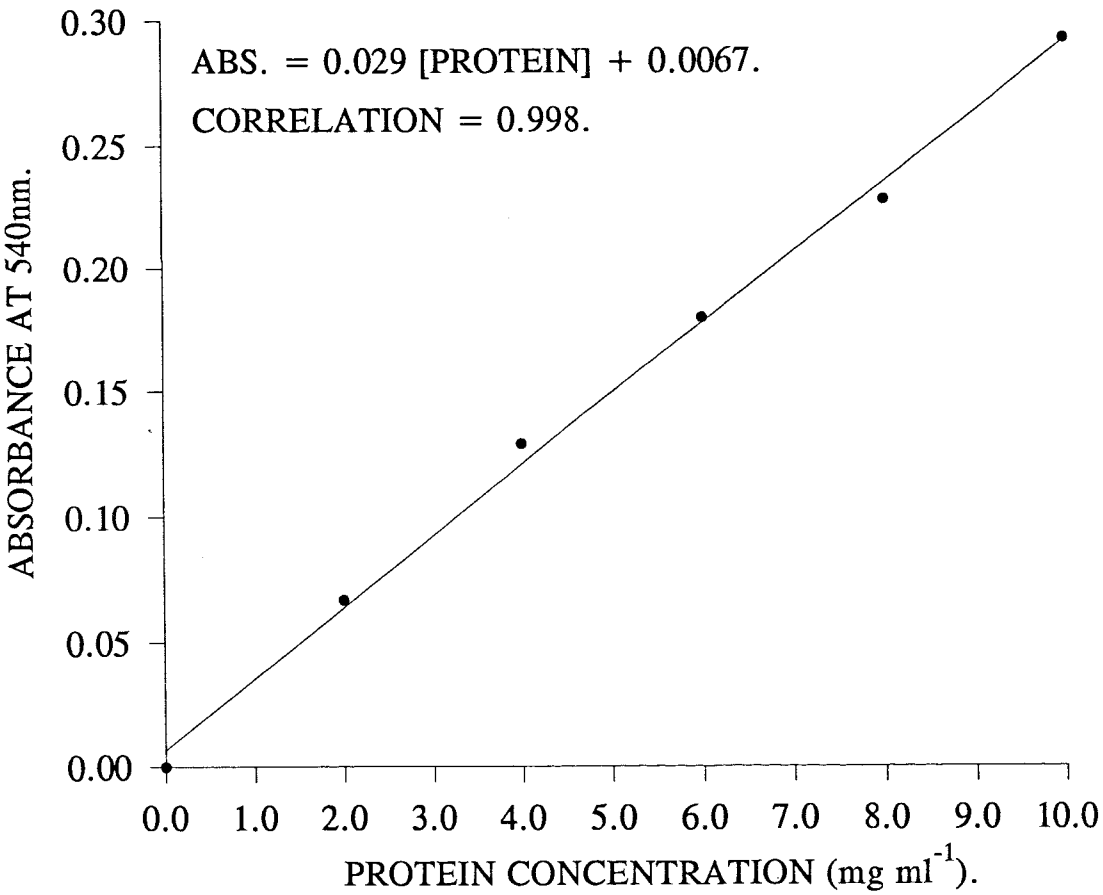

FIGURE A4.01 Standard curve for HLS protein determination confirming a linear relationship between protein concentration and absorbance at a wavelength of 540nm for protein concentrations in the range 0.0 to 10.0mg.ml^{-1}.

APPENDIX A.5 NEUTRAL RED RETENTION ASSAY REAGENTS.

Carcinus saline (Smith & Ratcliffe, 1978):

Used for diluting the neutral red stock solution and for incubating the haemocytes *in vitro* as well as for diluting the bacterial cultures prior to inoculation as described in chapter four.

A solution containing:

33.7g. Sodium chloride

0.94g. Potassium chloride

1.43g. Calcium chloride (2.83g. of the hexahydrated salt recommended by Smith & Ratcliffe, 1978).

5.39g Magnesium chloride hexahydrate

6.06g. Tris(hydroxymethyl)methylamine

- was made using approximately 700ml. of deionised water, the pH was then adjusted to 7.4 using a solution of 1M. hydrochloric acid before being made up to one litre using deionised water. The saline was then sterilised by autoclaving for twenty minutes at 120°C and stored at room temperature until required.

Neutral red solution (Korting *et al.*, 1994):

0.4g. neutral red powder (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) was dissolved in 100ml. of 100% ethanol and stored in an amber bottle at 4°C. Immediately before use this solution was diluted to a working concentration of 50μg.ml^{-1} using *Carcinus* saline.

Dye extractor (Borenfreund & Puerner, 1984):

A solution of 1% (v/v) acetic acid and 50% (v/v) ethanol was made using deionised water and stored at 4°C until use.

APPENDIX B

HISTOLOGICAL TECHNIQUES

APPENDIX B.1 STAINING PROCEDURE FOR THE DIFFERENTIAL HAEMOCYTE COUNTS (Sheehan, 1939; quoted in Gray, 1954).

a) A saturated solution of Sudan Black B in 70% ethanol was made.

b) A saturated solution of ethyl eosin in 70% ethanol was made.

c) A saturated solution of Methylene Blue in deionised water was made.

i) Air dried films were fixed for two minutes by immersing in absolute methanol.

ii) Film flooded with a) for thirty seconds and washed.

iii) Film dipped in 70% ethanol for one minute.

iv) Film immersed in b) for thirty seconds and washed.

v) Film immersed in c) for three minutes.

vi) Film rinsed and air dried.

APPENDIX B.2 FIXING AND STAINING OF GILL TISSUE (Bucke, 1989).

Davidson's Seawater Fixative:

Excised gill tissue was fixed in the following fixative before being stored in 70% ethanol prior to processing:

20% (v/v) Formaldehyde

10% (v/v) Glycerol

10% (v/v) Glacial acetic acid

30% (v/v) Absolute ethanol

30% (v/v) Seawater

Haematoxylin and Eosin Staining Procedure:

Harris' haematoxylin:

1g. Haematoxylin

10ml. Ethanol

20g. Potassium alum

200ml. Deionised water

0.5g. Mercuric chloride

8ml. Glacial acetic acid.

- the haematoxylin was dissolved in the alcohol and the alum was dissolved in deionised water by heating. The two solutions were then mixed, heated to boiling point and then the mercuric chloride was added. The mixture was rapidly cooled by immersion in cold water and when cold the acetic acid was added. The stain was stored in a glass stoppered bottle.

Acid/Alcohol Differentiator:

A solution of 1% (v/v) of hydrochloric acid in 70% (v/v) ethanol was made.

Eosin Counterstain:

1g. of eosin was dissolved in 100ml. of water and to it was added 1 small crystal of thymol.

Method:

1) Sections were taken to water.

2) Sections dipped into Harris' haematoxylin for 10 minutes.

3) Washed in running tap water for 5 minutes.

4) Sections differentiated in acid/alcohol for 10 seconds.

5) Washed under running tap water for 10 minutes.

6) Sections dipped in eosin for 2 minutes.

7) Sections removed and rinsed in tap water for 2 minutes.

8) Sections added to 70% (v/v) ethanol, removed and rinsed quickly.

9) Sections dehydrated rapidly in two changes of absolute ethanol, cleared

in xylene twice and mounted in DPX.

APPENDIX B.3 GRAM STAIN METHOD FOR BACTERIAL IDENTIFICATION (Stevens, 1982).

Lugol's Iodine:

A solution containing: 4g. iodine and 8g. of potassium iodide in 400ml. of deionised water was made and stored at room temperature.

Method:

1) Air dried bacterial broth smears were fixed by passing through a flame three times.

2) Films were stained with 1% (w/v) Crystal Violet for 15 seconds and the excess stain was poured of.

3) Films were flooded with Lugol's Iodine for thirty seconds and then the excess was poured off.

4) Films were then flooded with acetone for 2 seconds and immediately rinsed in deionised water.

5) Films were counterstained with Carbol Fuchsin for 20 seconds before finally washing with water and blotting dry.

APPENDIX C

BACTERIAL CULTURE METHODS AND BACTERIAL COUNTS

APPENDIX C.1 BACTERIAL CULTURE.

The original bacterial cultures were supplied by the Fish Diseases Laboratory, MAFF, Weymouth (Brooks, 1994). Listonella anguillarum strain 3.72 was used for experimental purposes and was cultured on both liquid and solid media based on a generalised seawater agar (table C1.01). Aquarium seawater was filtered through Whatman™ 0.1μm. cellulose nitrate filters to remove bacterial and other particulate matter; 250ml. of agar was usually used, which produced approximately 15-20 plates. Broth cultures were made as for the solid medium, without the addition of agar. The solutions were autoclaved at 120°C for twenty minutes to sterilise them before use.

Tryptic Soy Broth	1.0% (w/v)
Sodium Chloride	1.0%
triSodium Citrate	1.0%
Ferric Chloride	0.1%
Sucrose	2.0%
Agar	1.4%
Filtered Seawater	

TABLE C1.01 General seawater agar for bacterial culture.

The *Listonella* selective medium used was a thiosulphate-citrate bile salt agar

(Kobayashi et al., 1963; table C1.02). Although the prescribed method stated that this solution did not require autoclaving, the mixture was autoclaved (at 120°C for twenty minutes) to ensure a sterile medium. The seawater was filtered as for the general media, and again 250ml. was used to produce 15-20 plates.

*Bacteriological Peptone	1.0% (w/v)
Sodium Chloride	1.0%
triSodium Citrate	1.0%
Sodium Thiosulphate	1.0%
Sucrose	2.0%
**Ferric Citrate	0.1%
Ox Bile	0.8%
Yeast Extract	0.5%
Brom-thymol Blue	0.004%
Thymol Blue	0.004%
Agar	1.4%
Filtered Seawater	

TABLE C1.02 Thiosulphate-citrate bile salt sucrose agar for selectively isolating *Listonella* spp., as described by Kobayashi *et al.* (1963). * Tryptic soy broth, and ** Ferric Chloride used in this study.

The *Listonella anguillarum* (LAM) medium (table C1.03) described by Alsina *et al.* (1994) was used to identify bacteria isolated from the infected haemolymph of *Carcinus maenas* and grown up in general marine broth. Positive reactions for *L. anguillarum* were indicated by a colour change of the medium from blue to yellow

due to fermentation of the sorbitol producing a more acidic pH. Alsina *et al.* (1994) have indicated that this agar does not require sterilisation and in this instance the agar was not autoclaved after production as this would have destroyed the ampicillin. Table C1.03 indicates that the agar is made from deionised water and as before the water was filtered through Whatman™ 0.1μm. cellulose nitrate filters to remove any bacteria and particulates.

Sorbitol	3.75g.
Yeast	1.00g.
Bile Salts	1.25g.
Sodium Chloride	8.75g.
Cresol Red	10mg.
Bromothymol Blue	10mg.
Agar	3.75g.
Filtered Deionised Water	250ml.

TABLE C1.03 LAM agar (250ml.) used to specifically identify *Listonella anguillarum* from infected haemolymph samples.

Once the solution described in table C1.03 was produced it was mixed and boiled before being allowed to cool to 50°C. The pH was then adjusted to 8.6 ($\pm$ 0.2) using a solution of 6M. sodium hydroxide before 2.5mg. of ampicillin was added as an antibacterial to other species (*Listonella anguillarum* is resistant to ampicillin).

APPENDIX C.2 BACTERIAL COUNTS.

Bacterial broth was diluted to a 1:5000 concentration using filtered (through a Whatman™ 0.1μm. cellulose nitrate filter) seawater. A solution of 4,6-diamidino-2-phenylindole (DAPI) was added at a concentration of 1μl. per ml. This mixture was incubated at room temperature for fifteen minutes before filtering. The diluted broth was filtered through two filters - the top a Millipore™ 0.2μm. black cellulose nitrate filter, and the bottom a white Millipore™ 0.2μm. cellulose nitrate filter, used to support and protect the thinner black filter. When the solution was filtered the black filter was placed on a microscope slide with a drop of immersion oil on. A second drop of immersion oil was added to the top of the filter, and a coverslip added. The slide was examined under a Zeiss Standard Universal microscope with a mode II F1 vertical illuminator for fluorescence; barrier filters (53,0,0) and excitation filters (BG 38/2.5) were used. Five random fields were observed with an eyepiece counting grid, and the bacteria in each field counted. The concentration of bacteria was calculated from the equation:

$$\frac{A1 - A2}{V} \times F \times \frac{100}{30} \times D = Bacteria\ ml^{-1}$$

where:

A1 = Bacteria (from broth) counted per field (= 30μm^2).

A2 = Bacteria (from filtered seawater) per field (= 30μm^2).

V = Volume of sample filtered (5000μl).

F = Effective area of filter/Counting grid area (= 314.17μm^2).

D = Dilution factor (= 5000)

100/30 = Conversion factor to allow for the area counted.

APPENDIX D

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Circatidal rhythmicity in the activity of the phenoloxidase enzyme in the common shore crab, *Carcinus maenas*

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Specimens of the common shore crab, *Carcinus maenas*, were entrained to three different tidal regimes in a controlled laboratory environment using a computer-controlled "tidal tank". The first entrainment condition simulated a sinusoidal semi-diurnal tidal excursion of 0.90 m. The second entrainment condition, termed "exposed tidal", used the same tidal excursion as the first but incorporated 2 hr exposure to air around low water. The third system was atidal and acted as a control environment. After 9 days incubation, haemolymph was removed from individual crabs and assayed for phenoloxidase activity. Circatidal rhythmicity in the activity of the phenoloxidase enzyme cascade was demonstrated for the tidally entrained crabs, with peak activities occurring at low water. The converse phase was found for the tidally exposed crabs. The data presented are discussed in terms of the role of phenoloxidase in crustacean host defence and in relation to the location of phenoloxidase within the granulocytes of decapod crustaceans.

Key words: *Carcinus maenas*; Cellular communication; Circatidal; Host defence; Immunocompetence; Melanization; Prophenoloxidase: Rhythmicity.

Comp. Biochem. Physiol. 111B, 347–352, 1995.

Introduction

For neritic marine invertebrates, one of the most important facets of their ecology is the tidal nature of their environment and the regular changes in local conditions associated with water movement. Since the work of Naylor (1958) and Crothers (1968), who both demonstrated circatidal rhythmicity in the locomotor activity of the common shore crab, *Carcinus maenas* (L.), with increased activity levels coincident with periods of high tidal elevation, many papers have been published relating to the importance of tidal forcing on marine organisms (see review by Naylor, 1987). A large proportion of this type of research has been directed towards elucidating the nature of the endogenous clock or clocks involved in regulating locomotor activity (Reid and Naylor, 1989; Hunter and Naylor, 1993; Reid and Naylor, 1993).

By comparison, the influence of tidal forcing on invertebrate biochemistry has only received limited attention. Rajan *et al.* (1979), Williams (1985) and Delaney (1991) have all reported endogenous cyclical changes in the haemolymph sugar concentrations of *C. maenas*, with peak concentrations occurring at low water. Delaney (1991) has, in addition, reported a similar rhythmicity in the concentration of haemolymph hydrogen peroxide (an end-product of routine metabolism) that shows slight increases in concentration at low water.

The influence of tidal cycles on the internal "environment" of marine invertebrates has also been shown, particularly for molluscs, to include their immunocompetence. Most recently, Hawkins *et al.* (1993) have demonstrated tidally

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related changes in the immunocompetence of the American hard clam, *Mercenaria mercenaria*. Truscott and White (1990) first indicated a similar effect in crustaceans, in particular *C. maenas*, by demonstrating circatidal rhythms in total haemocyte count with maximum cell densities occurring during the high water period.

Cellular defence responses of crustaceans form only a part of a suite of activity which constitutes an immune system. Humoral activity is also of considerable importance and integral to the coordination of the two systems is the cellular based phenoloxidase enzyme cascade. The importance of this system was first highlighted by the studies of Söderhäll (1981) who demonstrated that β,1–3 glucans induced attachment of crayfish phenoloxidase to foreign surfaces such as glass and fungi when assayed *in vitro*; and Söderhäll and Ajaxon (1982) who showed that melanin precursors from the freshwater crayfish *Aphanomyces* spp. had fungitoxic properties when assayed *in vitro*. A large volume of data has since been generated concerning the activation of the phenoloxidase enzyme, the mechanics of the cascade, the potential roles of phenoloxidase constituting an immune response and its role in cellular communication (see reviews by Söderhäll and Smith, 1986; Söderhäll, 1992; Söderhäll *et al.*, 1994).

The aim of the present study was to further investigate effects of tidal forcing on the immunocompetence of crustaceans by determining the presence or absence of any entrainable circatidal rhythm in the activity of the phenoloxidase system in laboratory-maintained individuals of the common shore crab, *Carcinus maenas* and, in so doing, demonstrate any natural background variability in this potential index of organism condition.

Materials and Methods

Collection, maintenance and general experimental procedure

Specimens of *C. maenas* were collected using traps baited with fish and deployed sublittorally (approximately 1 m below chart datum, tidal range approximately 4 m) in the River Itchen within Southampton Water.

For the experimental incubation, only intermoult (stage C_4; Drach, 1939) male crabs, >55 mm carapace width, were chosen to avoid sex-linked differences in metabolism (Truscott and White, 1990; Williams, 1985; Delaney, 1991). Only those individuals that were apparently healthy, having no missing limbs, carapace lesions or any gross indication of shell disease, were selected. Prior to the period of experimental entrainment, all collected crabs were held in the aquarium for between 9 and 14 days in atidal conditions and continuous darkness. This allowed the individuals time to acclimate to the experimental milieu (salinity 33.0 ± (SD)0.2‰, temperature 16.3(SD)2.2°C); and was a sufficient period of time to ensure that any tidal or diurnal field-associated rhythms were lost (Williams, 1985; Delaney, 1991).

Three experimental programmes were set up—a tidal system in which animals were subjected to regular hydrostatic pressure changes. The crabs were maintained in continuous darkness in a "tidal tank" (Lockwood *et al.*, 1982) in which the water depth varied sinusoidally with a period of 12 hr 25 min but out of phase with the tides in Southampton Water. Each individual was held within a Netlon™ box on the base of the tidal tank. The tank allowed for a vertical water movement of 0.90 m, equivalent to a pressure change of 0.09 atm, which is sufficient to entrain cycles of locomotor and metabolic activity in *C. maenas* (Naylor and Atkinson, 1972; Williams, 1985; Delaney, 1991). The crabs did not receive aerial exposure during any part of the experimental schedule. The second system, designated exposed tidal, used the same tidal cycle of water depth but with the experimental animals held on a platform above the base of the tank, so that they were exposed to air for 2 hr around low water. The third system was atidal and acted as a control environment but, because of the constraints of space, the animals were allowed to move freely throughout the tank.

During the incubation, the crabs in all systems were fed every 2 days, but were starved for 24 hr prior to analysis. After a period of 9 days, crabs were removed from each system at regular intervals over a tidal cycle (low water to low water) and 0.6 ml haemolymph were withdrawn from each individual through the unsclerotized membrane between the coxopodite and basiopodite of the first pereiopod, using a sterile syringe and a 25-gauge needle. The animals were returned, after sampling, to their entrainment system and allowed to recover for at least a week before being sampled again. The experiment was run on a number of different batches of animals over a 2-month period and all animals were released at the end of their incubation into the River Hamble—approximately 12 km from their point of collection. During the experimental incubation no crabs died but occasionally the extracted haemolymph had a milky white appearance, indicative of a gross bacterial infection (Johnson, 1983); these haemolymph samples were discarded and the affected crab removed from the experiment.

Prophenoloxidase assay

Phenoloxidase activity was determined by adapting the methods of Söderhäll (1981), Smith and Söderhäll (1983), Söderhäll and Smith (1983) and Jackson *et al.* (1993). For this assay, all glassware and pipette tips were acid-washed to remove any trace oxidants before being rinsed twice in deionized water and once in Milli-Q™ water and sterilized by autoclaving at 120°C for 20 min to remove any bacterial films. All buffers and reagents were made up with Milli-Q™ water, as required.

Haemocyte lysate (HLS) preparation. The haemolymph samples of two crabs were pooled after 0.6 ml of haemolymph was separately withdrawn from each crab into a sterile syringe which contained 0.4 ml of ice-cold citrate EDTA buffer (pH = 4.6; Söderhäll and Smith, 1983) as an anticoagulant. Samples were then centrifuged at 450 *g* for 15 min and the pellet of haemocytes was washed twice, without resuspension, with 2 ml of ice-cold 0.01 M sodium cacodylate citrate buffer, pH = 7 (see Jackson *et al.*, 1993) before rapid freezing by immersion in liquid nitrogen, in 2 ml of 0.01 M sodium cacodylate buffer, pH = 7. Samples were stored frozen until the time of analysis when each was slowly defrosted and homogenized using a glass piston homogenizer on ice in a 5°C constant temperature room. Each sample was then centrifuged at 900 *g* and 3°C for a further 25 min to remove cell debris and the supernatant, designated HLS, was then assayed for phenoloxidase activity.

Activation and assay of phenoloxidase activity. Phenoloxidase occurs as an inactive zymogen within the granulocytes of crustaceans and so requires activating by an elicitor, in this case trypsin. Four hundred microlitres of HLS were incubated at 15°C with 400 μl of 0.1% trypsin (0.5 Anson units per gramme from beef pancreas) in cacodylate buffer. After 1 hr, 400 μl of dihydroxyphenylalanine (L-dopa, 4 g l^{-1} in Milli-Q™ water) was added to the activated HLS and the change in the absorbance of the sample was measured over the first 5 min. To take account of any background oxidation of the L-dopa by the cacodylate buffer or the inactive HLS, a control was prepared for each individual sample. The controls consisted of 400 μl of HLS, 400 μl of cacodylate buffer (to replace the trypsin) and 400 μl of L-dopa and they were treated in an identical manner to the samples.

Enzyme activity was expressed in units where one unit represented an increase in absorbance of the sample of 0.001 min^{-1} at 490 nm, all measurements were made on a Cecil CE 292 Digital Ultraviolet Spectrophotometer.

The protein content of the HLS was determined using the biuret method described by Raymont *et al.* (1964). For the determination, 2 ml of biuret reagent was added to 0.5 ml of HLS and incubated for 30 min at room temperature before being centrifuged at 450 *g* for 5 min. The optical density of the supernatant was compared with a bovine serum albumen standard (fraction V, 10 mg protein ml^{-1}) at 540 nm.

Enzyme activities were compared across the three entrainment conditions at low water and high water using one-way analysis of variance (ANOVA), where possible. In cases where the data were not normally distributed, the less stringent, non-parametric, Kruskal–Wallis test was adopted. Variation in enzyme activity over a tidal cycle for a single entrainment condition was examined using *t*-tests in those cases where the data were found to be normally distributed. Where parametric tests were not applicable the Mann–Whitney Rank Sum test was used.

Results

The activity of the immune defence enzyme phenoloxidase has been summarized for each exposure condition in Table 1 and in Fig. 1. From Table 1 it can be seen that phenoloxidase activity varied from 155.3 ± 49.3 to 424.6 ± 181.5 units/mg protein in the activated samples and from 2.5 ± 0.9 to 23.3 ± 7.4 in the blanks. Tidally entrained organisms demonstrated a decrease in enzyme activity/mg protein with increasing hydrostatic pressure whilst the opposite was evident in the tidally exposed individuals (see Fig. 1).

Table 1. Variation in phenoloxidase activity with entrainment condition and tidal state

	Entrainment condition								
	Tidal			Exposed tidal			Atidal/Control		
Tidal state	Sample	Control	*N*	Sample	Control	*N*	Sample	Control	*N*
Low water	424.6 ± 181.5*	7.7 ± 2.9	7	158.3 ± 49.1	2.5 ± 0.9	6	406.3 ± 190.9	14.6 ± 11.0	6
LW + 4	337.4 ± 123.7	8.7 ± 3.2	7	283.0 ± 63.5	8.6 ± 1.9	7	410.9 ± 134.5	9.4 ± 3.5	7
High water	212.9 ± 53.9	9.5 ± 2.1	7	348.3 ± 162.7	20.7 ± 9.4	7	173.4 ± 37.1	3.0 ± 1.0	7
LW + 8	155.3 ± 49.3	8.3 ± 4.3	6	237.1 ± 81.1	6.1 ± 2.4	7	250.1 ± 96.0	7.0 ± 3.1	9
Low water	371.0 ± 109.9	23.3 ± 7.4	9	229.9 ± 78.3	12.1 ± 2.6	8	210.3 ± 47.7	17.3 ± 5.9	9

*All values represent the mean ± 1 SE of *N* observations.

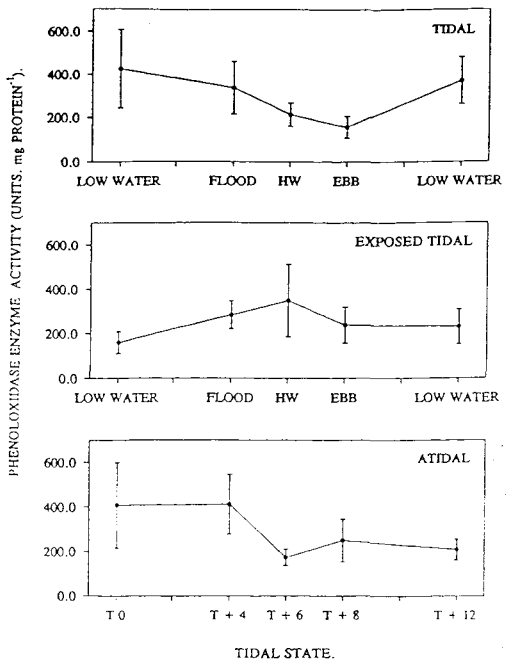

Fig. 1. Variation in phenoloxidase activity (trypsin activated samples only) with tidal state and entrainment condition, presented as mean values ± 1 SE. Sample sizes are as given in Table 1.

No significant differences in the level of phenoloxidase activity were found between the three entrainment conditions (one-way ANOVA or Kruskal–Wallis tests) or within a single exposure system (*t*-tests and Mann–Whitney Rank Sum tests). The opposite trends shown in the tidal and exposed systems are highlighted in Fig. 2 which shows the linear regression relationships between phenoloxidase activity and tidal elevation. There was a significant negative correlation between enzyme activity and water depth for the tidally entrained crabs (correlation coefficient, $r = 0.906$; $P = 0.034$) whilst the relationship was positive in the case of the tidally exposed crabs.

Control activity was very much less than for the activated samples and never represented more than 20% of the activity of the samples treated with trypsin elicitor. In both the tidally entrained and tidally exposed organisms, there was no significant correlation between tidal elevation and enzyme activity in the blanks.

Discussion

Circatidal variability in the activity of the phenoloxidase cascade has been demonstrated for the first time in this study. There was a significant negative correlation ($r = 0.906$) between enzyme activity and tidal height for the tidally entrained animals suggesting cyclical changes in immunocompetence. During "high water" events, the cellular store of inactive zymogen is reduced. In the past, high water has been reported by a number of workers as a period of increased locomotor activity amongst neritic decapod crustaceans (Naylor, 1958; Crothers, 1968; as reviewed by Naylor, 1988). Truscott and White (1990) similarly reported tidally associated rhythms in the total haemocyte count for freshly captured *C. maenas*, with peaks in haemocyte population occurring at high water. These workers suggested that "increased haemocyte numbers at high water may provide an enhanced immune capability during periods of high activity, when the risk of wounding is greater." Söderhäll and Smith (1983) have reported that the zymogen prophenoloxidase is activated only in the plasma. If more prophenoloxidase is released into the plasma during periods of high activity to enhance immunocompetence then the remaining cellular fraction (which is what is measured using this assay) would be reduced, as demonstrated in the present study.

Hawkins *et al.* (1993) have reported circatidal rhythmicity in a number of immune parameters in tidally entrained specimens of the American hard clam (*Mercenaria mercenaria*), including amoebocyte number and lysozyme activity, with peaks occurring at times of increased activity, including feeding. These workers also concluded

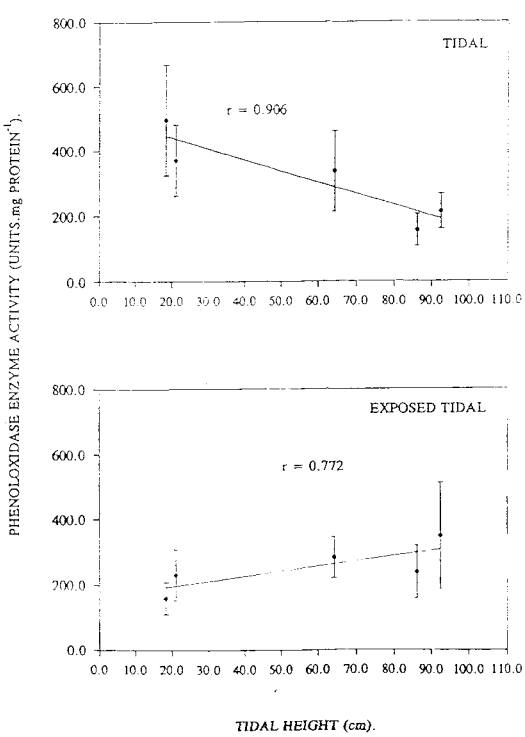

Fig. 2. First order linear regression relationships between phenoloxidase enzyme activity and tidal elevation for the tidal and exposed conditions. Data presented as for Fig. 1.

that this reflected an enhanced level of immunocompetence during a period of increased exposure to pathogens.

This study has formed part of a wider investigation into baseline variability in a number of potential metabolic and immunological indices of organism condition. Parallel studies of haemocyte population and haemolymph hydrogen peroxide and sugar concentration have been undertaken using the "tidal tank" (Lockwood *et al.*, 1982). Although using this laboratory-based system makes it possible to simulate tidal excursion whilst other environmental parameters remain constant, the tidal excursion is limited to a change in water level of approximately 1 m. Such a vertical displacement has been shown to be sufficient to entrain endogenous cycles of locomotor and metabolic activity (Naylor and Atkinson, 1972; Williams, 1985; Delaney, 1991), although clearly it is much less than animals typically encounter in the field.

For the exposed animals, the decreases in activity at low water may represent physiological adaptations to repeated bouts of exposure to air. It is conceivable that, during aerial exposure, the organisms may "switch off" energetically expensive metabolism, including the phenoloxidase pathway. This, however, remains a hypothesis that requires further validation.

A comparison of the results of Truscott and White's (1990) study with the present data would suggest that there is a negative correlation between total haemocyte population and phenoloxidase enzyme activity. However, as pointed out by Smith and Johnstone (1993), the units of measurement of phenoloxidase activity do not directly refer to cell number and there is no reason to expect a consistent relationship between cell population and enzyme activity. Phenoloxidase activity was measured in terms of a change in absorbance per milligramme of protein, which does not directly correlate with haemocyte population. Indeed, data collected simultaneously (Hauton, unpublished observations), which indicate maxima in the total haemocyte population towards high water for tidally entrained organisms and minima in the case of the tidally exposed specimens, demonstrate that the activity of the phenoloxidase enzyme does not consistently correlate with total haemocyte population. Similarly differential haemocyte counts made on the circulating population also show inconsistent relationships to the activity of the phenoloxidase enzyme.

The procedure used to purify a sample of HLS was adapted from a number of methods and was found necessary because of the experimental protocol adopted. Earlier methods have usually used much larger pooled samples of haemolymph (including Smith and Söderhäll, 1983; Smith and Söderhäll, 1991) which was not feasible in this study. The quick freezing of HLS in liquid nitrogen in this study was a necessary modification due to the constraints of the sampling procedure which prevented the analysis of HLS until the day after collection. Pye (1978) has successfully performed phenoloxidase analyses using reconstituted freeze dried haemolymph samples—the only refinement required in this present study was to freeze the haemocytes in the final homogenizing buffer ensuring that any lysis and subsequent enzyme release during freezing would not lead to a loss of the enzyme in discarded CAC—citrate buffer.

Questions must be raised regarding what "phenoloxidase activity" measurements actually represent. As discussed above, current models of the crustacean phenoloxidase system, such as that put forward by Söderhäll and Smith (1983), suggest that prophenoloxidase is released as a zymogen from the semigranular and granular cells into the plasma where it is activated by a serine proteinase. During the preparation of the haemocyte lysate supernatant (HLS) the plasma is discarded. As a consequence, the measure of phenoloxidase activity only indicates the total amount of prophenoloxidase being stored in the granulocytes and does not indicate any of the activated phenoloxidase that was present in the plasma. It would, perhaps, be more appropriate to measure any phenoloxidase activity in the plasma to indicate the active *in vivo* fraction. Söderhäll and Smith (1983), who were the first to isolate prophenoloxidase in the granulocytes, have, however, already reported an absence of activity in the plasma. This raises some problems relating to the apparent "disappearance" of the active phenoloxidase during assay. One possibility, suggested by Unestam and Nylund (1972), is that during the initial sedimentation of the haemolymph sample it is exposed to air whereupon, "the plasma rapidly melanises due to the action of polyphenoloxidase on substrates free in the plasma." To avoid the premature formation of melanin, White *et al.* (1985) adopted a new procedure overlaying the plasma with silicon oil.

It can be concluded from the data presented above and by those of Truscott and White (1990) that there is a circatidal rhythmicity in the immunocompetence of the shore crab, *C. maenas*. In light of this natural variability, it is evident that care must be exercised when interpreting field measurements of crustacean immunocompetence. It is clear that, for any measurements to be comparable, they must be taken at the same state of the tide so as to negate the influence of tidal forcing. Continuing studies

on this enzyme system will seek to re-examine the presence or absence of any plasma phenoloxidase activity so as to obtain an accurate indication of the active *in vivo* fraction.

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