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UNIVERSITY OF SOUTHAMPTON

The Aetiology of Environmental Stress
Responses and Disease in
Bivalve Molluscs

by

Jeremy David Brooks

A Thesis Submitted to the University of Southampton
for the Degree of Doctor of Philosophy

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September 1994

"...I was conscious of a passing pang for the oyster world,
feeling, - and I think correctly - that life for these
unfortunate bivalves must be one damn thing after another..."

P.G. Wodehouse

Jeeves and the Feudal Spirit. 1954.

UNIVERSITY OF SOUTHAMPTON
ABSTRACT
FACULTY OF SCIENCE
OCEANOGRAPHY
Doctor of Philosophy
THE AETIOLOGY OF ENVIRONMENTAL STRESS RESPONSES
AND DISEASE IN BIVALVE MOLLUSCS
by Jeremy David Brooks

The aim of this study was to provide a comprehensive set of quantitative and qualitative baseline responses at physiological, metabolic and immunological levels, in the Pacific oyster Crassostrea gigas (Thunberg), the European flat oyster Ostrea edulis (L.), and the Manila clam Tapes philippinarum (Adams and Reeve). The energetics of these species were compared across a matrix of temperature and salinity conditions. Field trials examined the effect of exposure of three O. edulis populations to infection by the protozoan parasite Bonamia ostreae, and enzyme electrophoresis investigated the genetic basis for any differences. Changes in immunocompetence were monitored from field samples and with controlled Vibrio anguillarum bacterial challenges. Haemolymph and haemocytic responses were recorded.

Filtration rate had the most significant effect on scope for growth (SFG) indices measured in all species. C. gigas showed a much wider range of filtration rates than O. edulis, and consequently had much higher SFG. Optimum environmental conditions for C. gigas occurred at 20-25°C and 19-25‰, compared with 20°C and 33‰ for O. edulis, and 15-20°C at 33‰ in T. philippinarum. Separate winter and summer physiological behaviour was detected in C. gigas and O. edulis, with the change occurring at 15°C and 10-12°C respectively. Body condition indices were inversely proportional to SFG and were probably related to the reproductive cycle. Temperature was shown to have the most significant influence on energetic factors, with salinity having little effect.

Field trials investigating Bonamia effects in three O. edulis populations found a significant, inverse size relationship with most of the physiological measurements. The largest animals, from Scotland, showed the lowest mortality, corresponding with increased energy input and decreased energy expenditure. The apparent resistance of this population probably arose from the fate rather than amount of assimilated energy. The Conwy population of O. edulis showed similar energy partitioning to the C. gigas and T. philippinarum animals, which also came from Conwy. Enzyme electrophoresis showed the three populations to be genetically very similar, with little genetic variation. Thus the differences in physiology were probably a result of phenotypic plasticity.

A phagocytic index on V. anguillarum uptake by O. edulis large granulocytes showed seasonal changes with higher levels in winter than summer. This was related to temperature stress inducing the physiological stress of reproduction. Hydrogen peroxide production varied similarly, but lysozyme activity was very variable. Bacterial challenge under seasonal conditions showed peroxide activity to increase significantly after 48 hours. Pathogen challenge in C. gigas under controlled environmental conditions also showed highest peroxide concentration at low temperature, with variable lysozyme activity. Hydrogen peroxide was considered the primary haemolymph defence mechanism.

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Drs. L.E. Hawkins and S. Hutchinson for giving me the opportunity of doing this study, and for their help and guidance throughout the course of the experimental work. My thanks also go to Dr. Hawkins and Dr. J.A. Williams for their constructive comments on the preparation of this manuscript.

I am very grateful for the supply of animals and information, and for the establishment of the field trials in the river Helford. In particular I would like to thank Mr. D. Bucke, of the MAFF Fish Diseases Laboratory, Weymouth, Mr. B.E. Spencer at the MAFF Fisheries Experimental Station, Conwy, and Mr. A.W. Berry of Knapdale Seafarms Limited.

I am indebted to the technical staff of the Department of Oceanography for maintaining equipment and allowing my practical work to continue without undue delays. In particular, grateful thanks go to Mr. D. Hutchinson, Mr. N. Jenkinson, Mr. G. Fisher, and Miss. K. Saull.

The help and support of my many friends without and within the Department of Oceanography has also been much appreciated, but I must particularly thank my colleagues from room 9 : Mr. Chris Hutton, for keeping my feet on the ground, Dr. Sonia Batten, for understanding and encouraging me, and especially Mr. Grant Rowe, who looked after me, sampled in all weather with me, and then kept my animals alive.

Finally, an extra-special thankyou to Dr. Corinne Jackson MRCP, who helped, cajoled, encouraged and supported me throughout the whole study, and deserves this thesis more than most.

POST-SCRIPT

My accident on 1st August threatened untold delays in completing this tome. However, friends and colleagues were quick to rally around and support me. I cannot express my gratitude enough to all the people who made my recovery and convalescence bearable, and almost enjoyable. Thankyou.

CHAPTER ONE

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Awareness of the pattern and processes causing diseases in marine bivalves, and especially oysters, has increased dramatically over the last 35 years (Fisher, 1988a) as the transfer of molluscs from one area to another has facilitated the spread of parasites and epizootic diseases. During this period scientific investigations have mainly concentrated on establishing parasitic life cycles, recognising and diagnosing disease, and describing patterns of disease. Research is now becoming directed at the need to understand thoroughly the host-parasite interactions and the environmental influence on hosts and parasites, or physiological and ecological responses to parasitism. For investigations to be made of the 'abnormal', a comprehensive knowledge of the 'normal' state is needed. This requires information about the organism's life cycle, such as physiological and metabolic behaviour, feeding strategies, predator/prey interactions, and reproductive strategies, as well as the effect of the environment on the animal.

Disease was defined by Kinne (1984) as a "*demonstrable negative deviation from the normal state (health) of a living organism*". This negative deviation implies an impairment which is quantifiable in terms of a reduction in the ecological potential of an animal, for example survival, growth, reproduction, energy procurement, stress and endurance (Stewart, 1991). The deviation may be functional, structural, or both, and may result from a single cause or from several potential causes acting in unison. Proliferation of pathogenic agents has placed a substantial burden on the lucrative oyster fishing industry and despite present-day knowledge of how causative agents occur and spread, disease still presents a serious problem to an industry dating back two thousand years. The need to establish the normal baseline physiological, metabolic, and immunological

parameters, before investigations of disease processes can be made, forms the underlying premise for the following study. Although many different molluscan species are exploited by the fishing industry, oysters were chosen as the main study species as these animals have a worldwide distribution, and form an important part of many countries' fishing programmes. *Ostrea edulis* (L.) and *Crassostrea gigas* (Thunberg) were the chosen species for study, the former because it has suffered high mortalities as a result of the parasite *Bonamia ostreae* (Pichot), the progress and pathogenesis of which has been widely described, and the latter because this species is not susceptible to *B. ostreae* and, consequently, has been introduced as a commercial replacement for *O. edulis*. *Tapes philippinarum* (Adams and Reeve) was also used for some of the work, as this not only allows the quantification of baseline responses of an increasingly important commercial species, but also increases the spectrum of bivalve species over which comparisons can be made.

1.2 The Molluscan Fishing Industry

The oyster mariculture industry in Europe can be traced back to Roman times when farmers built ponds to stock-pile their harvest, as well as attempting to collect spat on wooden fascines. Oyster fishing in oyster beds continued through the middle ages and the Renaissance period until the middle of the eighteenth century when fishing efforts had increased so much that overfishing of the animals became common, necessitating the introduction of regulations (Héral and Deslous-Paoli, 1993). In the mid-nineteenth century the richest oyster beds in Europe were to be found in the Thames estuary and the rivers of Essex (Utting and Spencer, 1992), with approximately 30,000 tonnes of oysters sold in Billingsgate Market alone in 1864 (Yonge, 1960).

From 1876 onwards, over-dredging led to a decrease in native oyster numbers, resulting in the import of *Crassostrea virginica* (Gmelin) oysters from North America. French farmers began to develop farming techniques, initially by enlarging the Roman wooden fascines, and then by evolving bottom and rack culture techniques (Héral and Deslous-Paoli, 1993). Between 1900 and 1940

British demand was largely met by imports, but in 1920/21 both British and French oyster stocks had severe disease problems caused by an unknown pathogen (Korringa, 1952). Settlement at normal levels was re-established in France by 1928, but British oyster beds were so severely depleted that imports from the Netherlands and France continued to increase to a peak of forty million animals in 1937. *C. virginica* was replaced from 1945 onwards by the Portuguese oyster *Crassostrea angulata* (Lamarck), although this species had been farmed in France since 1860 as an addition to the depleted native oysters - the 1920 production of *C. angulata* of 85,000 tonnes was almost equal to that of the flat oyster (Héral, 1986). An outbreak of a viral gill disease in the mid-1960s stopped production of *C. angulata* and resulted in the importation of adults from British Columbia, and spat from Japan, of a new oyster species *Crassostrea gigas*, the Pacific oyster. The appearance of two novel pathogens, in 1974 (*Marteilia refringens*) and 1979 (*Bonamia ostreae*), threatened to completely remove the native flat oyster from European waters, and switched the farming effort to the production of the Pacific oyster. Between 1981 and 1984 78,000-105,000 tonnes per annum of total living weight of *C. gigas* was produced in Europe compared with 4000 tonnes per annum for *O. edulis* (Food and Agricultural Organization, 1986, as cited in Héral and Deslous-Paoli, 1993).

Molluscan culture is commercially very valuable, for example with 1987/88 production in Australia valued at Aus\$65m for *Pinctada maxima*, Aus\$30m for *Crassostrea commercialis* and Aus\$8m for *Crassostrea gigas* (Australian Fisheries, 1992, as cited in Rohde, 1993), and a predicted harvest in 1991 of Can\$4m for *O. edulis* production in eastern Canada (Cross *et al.*, 1987). The most recent figures for the joint England and Wales oyster fishing industry show that in 1993 420 tonnes were landed with a value just under £740,000 (Lydon, *pers. comm.*). Thus, it is very important to acquire knowledge of host-parasite interactions, disease processes, and causes and methods of combatting disease, to try to preserve a profitable industry. For this reason the animals used in this study are *Ostrea edulis* and *Crassostrea gigas*, and also *Tapes philippinarum* which is increasing in commercial importance.

1.3 Study Animals

The general biology of oysters has been well documented in works by Korringa (1952), Galtsoff (1964), and Wilbur (1983), so only a brief introduction is given here.

1.3.1 *Ostrea edulis*

The native or European flat oyster *Ostrea edulis* is a larviparous, protandric hermaphrodite, with a lower fecundity than other oyster species (Héral and Deslous-Paoli, 1993). Individuals may change sex twice in a season, with individuals of both sexes present in a population at the same time, although male and female gametes do not ripen together in an individual. Gametogenesis and spawning are influenced by temperature, with populations throughout Europe thought to exist as a series of physiological spawning races (Korringa, 1957). *O. edulis* is a stenohaline species having an optimum salinity regime of 32-37‰ (Korringa, 1941); animals are able to grow well at 25‰, but in salinities less than 20‰ or greater than 40‰ the animal's growth and survivorship is seriously weakened. *O. edulis* has a 2000 mile range in Europe extending from Norway to Morocco, including the Mediterranean and Black Seas (Hidu and Lavoie, 1993) and has been introduced to the Pacific coast of North America, Nova Scotia, and Japan (Ahmed, 1975).

1.3.2 *Crassostrea gigas*

Genetic studies have suggested that the Pacific oyster *Crassostrea gigas* and the Portuguese oyster *Crassostrea angulata* are very similar or even the same species (Buroker *et al.*, 1979a; 1979b), but research into metabolic rates (Héral *et al.*, 1986), filtration rates (His, 1972), growth performance (His, 1972; Marteil, 1976; Bougrier *et al.*, 1986; Héral *et al.*, 1986), reproduction mode (Marteil, 1976) and resistance to disease (Combs, 1983), all of which show significant differences between the species, has established they are two species.

Crassostrea gigas has been cultured in Europe in place of *C. angulata* since the mid-1960s. The Pacific oyster differs from the native European species in that it is oviparous, with alternative protandry and high fecundity (Héral and

Deslous-Paoli, 1993). Individuals are either male or female for a season before changing sex for the following year. Reproduction is a function of age and temperature with a minimum temperature required for gamete release of 17 or 18°C. *C. gigas* is an extremely euryhaline species cultivated not only in oceanic waters but also in upper estuaries with a mean salinity of 15‰, or in salt marsh ponds at 45-50‰ (Héral and Deslous-Paoli, 1993).

1.3.3 *Tapes philippinarum*

Manila or small-neck clams, *Tapes (Ruditapes) philippinarum*, were introduced to Europe as hatchery techniques were developed, with small quantities of spat (500,000) and adults (50) introduced initially into France and Britain, making it easy to control any outbreak and spread of disease (Flassch and Leborgne, 1992). Although there were commercial problems linked with the ability of the species to reproduce naturally, the original adult stock of clams increased to a total European production of 600 tonnes in 1987 (Goulletquer *et al.*, 1989), and to 200,000 tonnes in 1990. Genetic selection is being used in an attempt to produce polyploids of a better 'quality', i.e. animals which are able to partition energy more into meat production and less into gametogenesis (Spencer, 1991).

The species is endemic to China, Japan and the Philippines, occurring in the muddy beach sand of the inter-tidal zone and shallow waters at the mouth of estuaries (Nie, 1993). Animals can survive in salinities of the range 16-36‰ although the preferred optimum range is 20-26‰. The preferred temperature range is 13-21°C, but clams will survive between 4 and 25°C (Bernard, 1983). *Tapes philippinarum* is dioecious, becoming sexually mature between one and three years of age. Animals will spawn either once or twice a year, depending on the location and environmental conditions, although the most northern populations will only spawn once, between April and October. Reviews of Manila clam biology and culture are given by Chew (1989) and Malouf and Bricelj (1989), and of the reproductive biology by Ponurovsky and Yakovlev (1992).

1.4 Diseases Associated with Oysters

All these animals carry a suite of other organisms, whether viruses, fungi or bacteria, and the composition is unique for each species. Opportunistic pathogens become effective in causing disease when host health or defence is compromised by problems such as nutritional deficiencies, adverse environmental conditions, or excessive densities of animals. Bonamiasis is possibly the best documented example of how introductions and transfers of commercial species cause the spread of disease.

Bonamia ostreae (Pichot) was originally diagnosed as the pathogen responsible for mass mortalities in Brittany, France (Pichot *et al.*, 1979; Comps *et al.*, 1980). Movement of infected animals facilitated the rapid spread of the disease around Europe, where diseased animals were discovered in the Netherlands (van Banning, 1982; 1987), Spain (Polanco, 1984), Ireland (McArdle *et al.*, 1991) and the U.K. (Bannister and Key, 1982; Hudson and Hill, 1991). *B. ostreae* has also been reported on the west coast of the U.S.A. (Elston *et al.*, 1986; Friedman *et al.*, 1989) from where it is thought to originate (Katkansky *et al.*, 1969; Elston *et al.*, 1986). The presence of a *Bonamia* sp. in New Zealand (Dinamani *et al.*, 1987; Hine, 1991) indicates the worldwide importance of this epizootic disease. The pathogen is a species of unknown phylogenetic affinities (Perkins, 1988) although it is believed to be a haplosporidian (Rohde, 1993) which causes haemocytic parasitosis (Balouet *et al.*, 1983). It has been found in haemocytes and extracellularly in gill epithelia (Bucke and Feist, 1985; Chagot *et al.*, 1992) but it is only very recently that a life cycle has been tentatively suggested (Montes *et al.*, 1994). Infection appears to occur directly from oyster to oyster (Bucke, 1988; Perkins, 1988) with 80-100% mortality in six months (Balouet *et al.*, 1983; Elston *et al.*, 1986) and although highest infection occurs during the warmer summer months, low temperature (4-5°C) does not inhibit transfer in winter (Tigé and Grizel, 1982). Stress appears to be an important factor in lowering resistance to the parasite and is probably the major reason why cultured oysters suffer greater mortalities than natural populations, as they are handled more throughout their life (Stewart, 1991).

The devastating occurrence of the paramyxean parasite *Marteilia refringens*

in oyster beds in the early 1970s (Grizel *et al.*, 1974) was compounded by the lack of knowledge of the disease, its life cycle, and its effects on the host. The impact of bonamiasis enabled some of the problems in combatting parasites to be resolved (Grizel *et al.*, 1988); preventative measures were implemented more quickly, disease recognition was developed, and tests for breeding resistant populations were established (Elston *et al.*, 1987b). A thirteen point guideline was established for farming molluscs in the U.K. (Hudson and Hill, 1991), reducing infections to less than 10% in many areas. However, it became more obvious that stress factors were important in lowering disease resistance as *O. edulis* was significantly affected by dredging, handling, and exposure to air, low temperatures and fluctuating salinity.

Crassostrea gigas, although unaffected by *Bonamia ostreae* or *Marteilia refringens*, has been shown to be susceptible to its own unique disease problems. Summer mortalities, i.e. greater than 30% death within a population, have been recorded regularly since the late 1950s on the Pacific coast of U.S.A. and Canada (Beattie *et al.*, 1988). Mortalities are usually found in areas characterised by their high turbidity and increased dinoflagellate production (Beattie *et al.*, 1988), but, perhaps more importantly, the mortalities coincided with increased water temperature (c. 20°C) and when the oysters were spawning (Lipovsky and Chew, 1972). Further studies isolated *Vibrio* spp. of bacteria associated with the event (Grischkowsky and Liston, 1974), which were probably secondary to the likely causative agent identified as a *Nocardia* sp., an actinomycete-like bacterium (Friedman and Hedrick, 1991). Although these bacteria were the likely pathogenic cause, their effects would have been reduced without the stress involved with using energy in gonad maturation and spawning, rather than in defence mechanisms.

Crassostrea gigas has also suffered from a microcell disease - *Mikrocytos mackini* or Denman Island Disease (Bower, 1988; Farley *et al.*, 1988). The first occurrence of this problem was in 1960, but outbreaks have been recorded regularly since then. Quayle (1961; 1969; 1982) observed that common features in each outbreak included greatest mortality in oysters at the low tide level, on a seasonal basis - March to June only, as temperatures rise - and with younger, less

mature animals showing a decreased susceptibility compared with older animals. Knowledge of this sort can help in producing an effective management plan for the culture of animals with minimal disease prevalence, for example planting or harvesting times and densities, and depths of planting with the younger oysters at the low tide mark. Management strategies have also been implemented for controlling *Haplosporidium nelsoni* (Ford and Haskin, 1988) and *Perkinsus marinus* (Andrews and Ray, 1988) in *Crassostrea virginica*. However, it is clear from these reports that stress, whether environmentally or anthropogenically induced, is very important in determining susceptibility to disease and consequently recognition of the interactions between the environment and the host's physiology, immunology and metabolism is necessary in managing the problem.

1.5 Quantifying Stress

No habitat is totally benign and changes in the physico-chemical regime can occur in different parts of the ecosystem, having direct or indirect effects on the study organism. The adaptability of an organism to these sorts of changes is defined by its capacity to adjust its physiology to operate with optimal efficiency in a variable environment (Bayne *et al.*, 1985), although this definition may be extended to include an animal's defence processes and metabolism. Change in the environment will induce a potential stress factor, defined by Brett (1958) as "*a state produced by an environmental 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*". Bayne (1984) modified this definition slightly and used stress to refer to "*the environmental stimulus (rather than to the physiological state that represents the response) which, by exceeding a threshold value, disturbs normal animal function*".

Experimental procedures which measure the effects of environmental changes within the normal experience of an animal can help in understanding the animal's distribution in nature, and its limits of tolerance to natural change. This

provides a quantitative indicator of ecological fitness, for example in response to pathogens, useful in the management of stocks and even for the selection of particular phenotypic traits conferring advantage over others. Sparks (1993) indicated that this sort of knowledge is required before and during investigation of invertebrate diseases, at two levels. At the organismic level the normal life cycle of the host animal is needed, i.e. normal patterns of growth, maturation, reproduction and senescence. An understanding of the normal histology of the animal is needed so that normal architecture can be distinguished from that which is diseased. The process of inflammation and wound repair, and the post-mortem changes occurring with death, should also be known. At the population level data are needed about normal mortality patterns, the normal microbial flora and parasite load associated with a particular species, and the environmental tolerances of the species under examination.

Sparks (1993) states that this basic information is often, at best, sparse, and at worst, absent altogether, leaving three basic avenues of scientific exploration. The first is to initiate thorough studies of the normal prior to delving into the abnormal. However, this is a lengthy and time-consuming procedure and often not practicable. Secondly, the investigator can plunge headlong and unprepared into a full-scale investigation of the alleged mortality, but this is an undesirable method which may result in mis-interpretation of data. Finally, thorough controlled studies of the normal may be attempted concurrently with separate, primarily field investigations of the diseases of the population or species in question, and it is this method which was adopted for the present study.

1.6 Indices of Stress

To gain the most complete understanding of the causes and consequences of diseases in molluscs, techniques for measuring physiological change must be employed in conjunction with more routine pathological and histological methods (Newell and Barber, 1988). By using other measurements such as metabolical, immunological and haemocytic indices, a comprehensive data set can be established characterising the 'normal' behaviour of an animal or a population

under a given set of conditions. A physiological approach has already been used to study anthropogenic pollutant effects on marine fauna (Bayne *et al.*, 1985) and the importance of environmental factors influencing molluscan physiological processes is well documented (Newell, 1979; Bayne and Newell, 1983), although much less well known when assessing host-pathogen interactions. This study aims predominantly to examine the effects of natural environmental stressors, and consequently anthropogenic stressors and their effects have largely been ignored. Although these are of considerable importance in increasing disease susceptibility, it is vital to quantify normal physiological functioning first in order to understand how an animal's responses are changing to other stressors. Reviews are given by Cheng (1993) and Sindermann (1993) on non-infectious diseases and pollutant problems in molluscs.

1.6.1 Physiological Methods

A number of different indexation methods have been employed for the present study. The scope for growth (SFG) method, described by Bayne *et al.* (1985), is especially useful as it measures energetic changes giving a quantitative indication of energy available for somatic growth or gametogenesis. The incorporation of filtration, respiration and excretion rates, and absorption efficiency into a single overall index also shows which processes are affected by stress or disease, and how these affect the animal as a whole.

Filtration rate is frequently affected early on by parasites as water-borne pathogens are taken in in a ventilatory current created by cilia in the gills (Galtsoff, 1964; Jørgensen, 1981; 1990). Parasitic lesions which form on the gills stimulate mucous production which interferes with cilia activity and reduces filtration efficiency (Jørgensen, 1990). Gill disease in *Crassostrea angulata* is caused by a virus which eventually results in the destruction of complete gill filaments. Severity of the disease has been correlated with tissue weight loss, and was attributed to depression of feeding activity (His, 1969). Measurements of respiration rate and nitrogenous excretion provide further physiological information which can be incorporated in the SFG calculation. The ratio of oxygen uptake to nitrogen excreted can also be used as a physiological index of stress as

this represents protein catabolism - a low ratio demonstrates a high level of protein turnover.

Unfortunately, the SFG energy budget only measures short-term changes, and does not indicate whether somatic or gametic production, or both, is affected. Cheng and Burton (1965) showed female bivalves infected with *Bucephalus* sp. had smaller ova than non-infected animals, primarily as a result of reducing the egg nutrients which will ultimately reduce the larval viability (Bayne *et al.*, 1975; 1978). Using SFG this reduction would have been indistinguishable from a decrease in glycogen and lipid storage reserves, as occurs in *C. virginica* parasitised by *Bucephalus* sp. (Cheng and Burton, 1966). This problem is overcome to some extent by the additional use of a body condition index (BCI) where the dry tissue weight:shell cavity volume is compared. Kent (1979) showed that *Polydora ciliata* associated with *Mytilus edulis* would produce a significant reduction in the condition index of the mussel.

Long-term seasonal measurements have been made examining environmental and anthropogenic influences on *Crassostrea virginica* production, and how this knowledge can be used to predict yields (Allen and Turner, 1989; Abbe, 1992); the effects of climate change on a seasonal basis have also been monitored in *C. virginica* with respect to yearly changes in parasitism by *Perkinsus marinus* (Powell *et al.*, 1992; Wilson *et al.*, 1992). Seasonal changes in *Crassostrea gigas* growth, condition index, and survival time have been quantified (Brown and Hartwick, 1988a; 1988b), as has the biochemical composition of blood serum in *Mytilus galloprovincialis* (Robledo *et al.*, in prep).

1.6.2 Haemocytic and Immunological Indices

Read (1958) defined disease susceptibility or insusceptibility as to whether a host could fulfil the nutritional needs of a parasite, and observed that resistance could be independent of susceptibility. Factors which influence the host's resistance can be divided into two sources (Feng, 1988). Extrinsic factors are usually fortuitous and ecological in nature, and are associated with climatic changes, density dependent factors and food preference of the host. Intrinsic factors are host-specific and consist of innate resistance, i.e. anatomical,

physiological, biochemical and behavioural traits effectively preventing the host from acquiring infections, and acquired resistance as a result of exposure to infectious agents. Both of these responses may consist of cellular and humoral factors, although humoral responses can ultimately be traced back to the cellular level.

Haemocytic indices are an alternative method of indexing stress and provide information on the immunological status of an animal. Natural environmental variation is known to play an important role in the defence response of many animals (Fisher, 1988b) but it is particularly true of marine and estuarine bivalve molluscs which are poikilothermic osmoconformers (Galtsoff, 1964; Shumway, 1977). With a partially open circulatory system the haemocytes are exposed to the temperature and salinity changes occurring in the environment, which consequently has an effect on processes such as endocytosis, inflammation, wound repair, encapsulation, diapedesis, and enzyme encapsulation (Fisher, 1988b). These effects will ultimately be a major factor in determining susceptibility to parasitism or other associated problems.

The different molluscan haemocyte types have been reviewed by Cheng (1981), Fisher (1986) and Auffret (1988). It is these cells which are responsible for the inflammatory process and wound healing, without which parasitism would result in necrosis of the host tissues. In phagocytosis a distinct sequence of events can be identified, with particle recognition followed by adherence, uptake, destruction and disposal of the invading body (Feng, 1988). Intracellular degradation occurs by a range of lysosomal enzymes, although extracellular digestion by elevated lysozyme has been observed in haemolymph samples of *Mercenaria mercenaria* (Cheng *et al.*, 1975; Foley and Cheng, 1977; Mohandas *et al.*, 1985). Phagocytosis requires the movement of cells - frequently termed amoebocytes for the manner of their locomotion - the spreading and rate of locomotion of which can be quantified as a haemocytic index. Fisher (1988b) demonstrated high temperature stress when measuring locomotion in *Crassostrea virginica* haemocytes. This was further compounded by low salinity (6‰) which affected volume regulation at the expense of other cell functions.

Stimulation of phagocytic activity helps to promote humoral responses to

pathogen challenge. One of these methods is by eliciting a respiratory burst with the production of superoxide anion, hydrogen peroxide, hydroxyl radical, and singlet oxygen. All these are potent microcidal agents and have been demonstrated in *Patinopecten yessoensis* (Nakamura *et al.*, 1985) and *Lymnaea stagnalis* (Dikkeboom *et al.*, 1985), with the respiratory burst shown by chemiluminescence experiments (Chagot, 1989). The production of lysozyme as a humoral defence mechanism against pathogens has been demonstrated in both the haemolymph and haemocytes of *Crassostrea virginica* (McDade and Tripp, 1967; Feng *et al.*, 1971; Rodrick and Cheng, 1974; Cheng and Rodrick, 1975); this extracellular digestion is associated with degranulation of the haemocytes. Temperature and salinity effects, together and in isolation, and tidal effects on humoral hydrogen peroxide and lysozyme have been shown in *Ostrea edulis* (Hawkins and Hutchinson, 1990) and *Mercenaria mercenaria* (Hawkins *et al.*, 1993a).

1.7 Stress Inducing Factors

Environmental stress, as has already been indicated, has serious implications in susceptibility to disease in marine bivalves. Stress may manifest itself in several different forms but the largest stress effects are created by temperature and salinity changes. Bivalves acclimate to seasonal changes in temperature by altering feeding and respiration rates in order to maintain a stable scope for growth (Newell, 1979). However, the summer mortalities in *Crassostrea gigas* have been shown to occur during periods of increased water temperature (Lipp *et al.*, 1976; Beattie *et al.*, 1988). *Vibrio* spp. counts in *C. gigas* from Puget Sound, Washington U.S.A. were directly correlated with water temperature (Baross and Liston, 1968; 1970), and bacterially mediated mortalities in the same oyster species have also been shown to be proportional to water temperature. High temperature has also been shown to be responsible for parasitism and mortality resulting from *Perkinsus marinus* (Mackin, 1962).

Salinity fluctuations also induce stress in molluscs, with many species enduring wide ranging conditions from the upper estuary to the marine environment. All animal tissues are open to such fluctuations so it is not only the

haemocytes which have to maintain cellular competence. Over a tidal cycle oyster metabolism may change from being active at high tide to stopping totally at low tide (Héral and Deslous-Paoli, 1993).

Hypoxic stress is induced in animals exposed at low tide, when they are forced to keep their valves shut tight, preventing aerobic respiration. Under anaerobic conditions, oyster metabolism has been shown to alter metabolite build-up, especially that of succinate (Hammen, 1969; Mustafa and Hochachka, 1971). Chronic anaerobiosis eventually causes lactate to accumulate, which is toxic to visceral tissues. Laird (1961) cited environmentally induced anoxia as responsible for increased disease susceptibility in *O. edulis* and *C. virginica*.

Nutritive stress can be induced by low quantity or poor quality of food, as occurs seasonally in coastal areas (Widdows *et al.*, 1979; Berg and Newell, 1986). It is the amount of energy assimilated from the food which has a direct bearing on the scope for growth of an animal, and also on the amount of energy which is available for defence mechanisms. Bayne *et al.* (1979) showed *Mytilus edulis* infected with the parasitic copepod *Mytilicola intestinalis* could cope with infection if food availability was high. Stress may also be induced by the life cycle of an animal, for example during spawning or gametogenesis. Post-spawning animals have depleted energy reserves (Bayne *et al.*, 1976; Barber and Blake, 1981; Bayne *et al.*, 1982) and although the mass mortality suffered by *C. gigas* in Matsushima Bay, Japan was characterised by a fatal infection bacteraemia (Elston *et al.*, 1987a) its occurrence was attributed to physiological stress as a result of spawning and high water temperatures (Mori *et al.*, 1965a; 1965b; Imai *et al.*, 1968; Beattie *et al.*, 1988).

These examples of stress inducing factors, as well as the way in which they affect the host, suggest that there is a continued need to measure the links between environmental stress levels - scope for growth, body condition index, and other indices of stress - and resistance to parasitism and disease - measured immunologically, with incidence and mortality recorded.

1.8 Aims

The sessile nature of oysters means they are chronically exposed to environmental fluctuations and without basic knowledge of the changes in animal biology associated with a changing environment, it is, at best, extremely difficult to draw definite conclusions as to the causes and effects of disease in host organisms. The rationale for scientific investigations indicated by Sparks (1993), together with the recommendation by Couch (1985) that "*future monitoring studies include careful study of tissue and physiological changes for indication of...stress*", provide the *raison d'être* for this study.

The major aim of this investigation was to provide a comprehensive data-set of quantitative and qualitative baseline responses at physiological, immunological, and to a lesser extent metabolic levels. The major study animal used for these assays was the Pacific oyster *Crassostrea gigas*, and by using a matrix of combinations of temperature and salinity measurements, the energetics of the species was determined. This complemented work performed on *Ostrea edulis* by Rodhouse (1977), Newell *et al.* (1977), Buxton *et al.* (1981), and Hutchinson and Hawkins (1992), and provided comparative data on two commercially important species. Field trials established by the Fish Diseases Laboratory, MAFF, Weymouth contributed data for three populations of *O. edulis* before and after exposure to *Bonamia ostreae* in the natural environment, and with the use of electrophoresis, the genetic basis for any differences was investigated.

Immunological techniques were used to assess seasonal variation in field samples and together with controlled bacterial challenges, with *Vibrio anguillarum*, under different environmental conditions, changes in immunocompetence were monitored. Limited investigations into the physiology and immunology of another commercially important bivalve, *Tapes philippinarum*, were included where possible as a complement to the work of Goulletquer *et al.* (1989), and as a comparative species with the oysters.

CHAPTER TWO

PHYSIOLOGICAL MEASUREMENTS

IN *Crassostrea gigas*

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PHYSIOLOGICAL MEASUREMENTS IN *Crassostrea gigas*

2.1 INTRODUCTION

The introduction of novel species brings about further considerations with the possible introduction of new disease and stress problems. *Crassostrea gigas* is fundamentally different from the native flat oyster *Ostrea edulis* in its mode of reproduction as it is non-incubatory or oviparous and, as such, the eggs discharged into the water column are fertilised outside the animal. In contrast *Ostrea* species are larviparous and fertilise the eggs in the gill cavity before brooding larvae for upto two weeks and ultimately releasing fully developed veligers into the natural environment. The sexes of non-incubatory oysters are separate although unstable and once a year a certain percentage of the population will change sex, usually after spawning. A comprehensive account of Ostreidae biology, with particular reference to *Crassostrea virginica*, was given by Galtsoff (1964) and remains a definitive text on this family of bivalves. The destruction of the flat oyster fishing industry around Europe, by the parasite *Bonamia ostreae*, has led shellfish farmers to exploit alternative bivalve species. The introduction of the Pacific oyster to northern latitudes has proved commercially successful and this species is now farmed extensively throughout Europe.

2.1.1 Introduction of *Crassostrea gigas* to Britain

The introduction of most non-indigenous bivalve species into U.K. waters has been deliberate, usually for commercial exploitation. Before 1960 there was no legislation to control import and deposition of oyster seed, but growing awareness of the risks in introducing potential diseases, pests and parasites into native populations ended this trend. In 1965 the Molluscan Shellfish (Control of Deposit)

Order, which was strengthened as the Molluscan Shellfish (Control of Deposit) Order 1974 and amended in 1983, tightened restrictions on the movement of oysters around the coast of Britain. These control measures helped to prevent the spread of *Bonamia ostreae* after its initial discovery in 1982.

Crassostrea gigas was first introduced into Britain in 1965 (Walne and Spencer, 1971; Utting and Spencer, 1992). Its rapid growth rate and subsequently discovered immunity to *B. ostreae* made it an ideal commercial replacement for the native oyster *O. edulis*. Current production of the Pacific oyster in this country is only 600 tonnes per year but this figure is expected to increase by 30% per year as the use of TBT in anti-fouling paints is restricted. Although *C. gigas* requires high temperatures for larval recruitment, isolated incidents of naturally recruited spat have been reported in this country from unusually warm, shallow, enclosed bodies of water, such as Emsworth Harbour (Utting and Spencer, 1992). The commercial production of *C. gigas* is supplemented by hatchery produced seed.

2.1.2 Previous Studies

In general *C. gigas* is more tolerant of dilute sea water than *O. edulis*, which prefers high salinity, less turbid water (Galtsoff, 1964), but the extent to which these species differ is still unknown. The mechanism of transfer and the life-cycle of *B. ostreae* are still largely unidentified and, as with many diseases of bivalves, the conditions under which animals are most susceptible remain mostly anecdotal. Awareness of how different environmental conditions combine to induce stress in animals has led to the necessity for studies of an animal's baseline physiology, before definite conclusions can be drawn as to how disease or infection are acting.

Limited investigations have been made on some of the effects of environmental parameters on aspects of physiology in a number of bivalve species - in *Crassostrea virginica* the effects of salinity variations have been measured on enzyme activity (Ballantyne and Berges, 1991), whole and partial sections (Paparo, 1989), osmotic properties (Ballantyne and Moyes, 1987), and the effects of salinity in combination with other environmental parameters has been examined on

metabolism (Eberlee *et al.*, 1983; Ballantyne and Berges, 1988; Widdows *et al.*, 1989) and haemocytic competence (Fisher and Tamplin, 1988; Chu and Lapeyre, 1989; Fisher *et al.*, 1989; Larson *et al.*, 1989; McCormickray and Howard, 1991). In *C. gigas* the combinations of salinity, temperature and other environmental variables have been studied but with particular emphasis on the growth of larvae (His *et al.*, 1989; Zimmerman and Pechenik, 1991), growth of adult animals (Levitt, 1987; Brown and Hartwick, 1988a; 1988b) and on metabolism (Jeong *et al.*, 1991; Seaman, 1991). More limited studies of temperature and salinity effects have also been performed on the oysters *Crassostrea commercialis* (Rowse and Flatt, 1984) and *Crassostrea rhizophorae* (Castro *et al.*, 1985; Dossantos and Nascimento, 1985; Littlewood, 1989).

Unfortunately, no study gives a comprehensive account of the physiological response of *C. gigas* across a combination of temperature and salinity, which is necessary to quantify parasitic effects. Hutchinson and Hawkins (1992) quantified the ecophysiology of *O. edulis* over a matrix of temperature and salinities, and indicated the importance of considering environmental effects on an organism before a full understanding of disease susceptibility could be reached. This study represented a unique, integrated approach to the characterisation and application of bivalve stress biology by including fundamental measurements of physiological, metabolic and immunological responses. Goulletquer *et al.* (1989) calculated an energy balance for the manila clam *Ruditapes philippinarum* based on its ecophysiology at various temperature and salinity combinations. However, this study was not as comprehensive in its determination of baseline reactions as the previous study as it was limited to only a few temperature variables. An aim of the present investigation was to quantify the basic physiological biology of the Pacific oyster *Crassostrea gigas* to a matrix of temperature and salinity as a pre-requisite for future studies of the effects of anthropogenic and natural stressors.

2.2 MATERIALS AND METHODS

2.2.1 Collection and Maintenance of Animals

Crassostrea gigas of approximately 15-25 g wet shell weight were provided by the MAFF laboratory, Conwy, and were maintained in flow-through tanks with aeration, at ambient water temperature (13-19°C) and salinity (31-33‰).

Acclimation to the different experimental temperature/salinity combinations was performed slowly, over a period of 48 hours. This procedure was controlled by micro-processor. Animals were subsequently kept at this temperature for seven days before any experimental analysis was performed. Although the matrix of variables was measured over a period of eighteen months, seasonal effects were avoided by this acclimation period.

The animals were fed daily a mixed algal ration of the diatoms *Phaeodactylum tricornutum* and *Chaetoceros calcitrans* and the dinoflagellates *Tetraselmis suecica* and *Isochrysis galbana*. The method for this algal culture is given in Appendix A1. This dietary regime was also supplied to animals in acclimation and experimental tanks. A mixed algal diet was used so as to more closely resemble a natural seston, following the observations of Doering and Oviatt (1986) and Bayne *et al.* (1987); the diet also provided a measurable inorganic component that is needed to calculate absorption efficiencies (Jørgensen, 1990). It was assumed that there was no preferential uptake of algal species as it is known that there is no size selection of particles up to 120 µm in a number of bivalve species (Yonge, 1926; Foster-Smith, 1975). Animals of approximately the same shell size were used to minimise size related effects. Each experimental group consisted of 12 animals; all animals for scope for growth experiments were cleaned of epifauna and individually numbered with waterproof paint. The individual components of scope for growth were measured for each individual animal, using modifications of the procedures given by Bayne *et al.* (1985). At the end of the experiments, each animal was sacrificed for dry- and ash-weight measurements, before individual scope for growth indices were calculated.

2.2.2 Physiological Measurements

FILTRATION RATE

Filtration rates were measured using a specially constructed upwelling tank. Six animals were kept in individual chambers, with a seventh chamber kept empty as a control. Animals were allowed to acclimate to this system for 48 hours, with minimal disturbance, in order to eliminate inaccurate measurements. The water temperature and salinity conditions were controlled by micro-processor. Algae were added at a constant rate (5-10 ml min⁻¹) by a peristaltic pump; the concentration of cells was maintained at approximately 25 000 cells per ml. The water flow through the system was ≥ 250 ml per minute except at the very lowest temperature where the rate was reduced to prevent warming the water. Water samples from the overflow pipes were taken for each chamber, with not less than 30 minutes between each sampling; the first sample was taken after a minimum one hour from the experiment starting. Counts of the algal cell numbers in each water sample were made with a Coulter TAPI™ electronic particle counter. Flow rates for each chamber were measured by the time taken for 100 ml to overflow. The filtration rates of individual oysters were calculated from the difference between numbers of algal cells in the flow from experimental chambers and the numbers from the control chamber. The equation for clearance rates was taken from Hildreth & Crisp (1976) :

$$\text{Clearance Rate (l h}^{-1}\text{)} = \frac{C_i - C_o}{C_i} \times F_r$$

where C_i = inflow particulate concentration

C_o = outflow particulate concentration

F_r = flow rate for each animal (l h⁻¹)

ABSORPTION EFFICIENCY

Absorption efficiency was determined using methods adapted from those described by Conover (1966). Samples of faeces were collected from each animal, not more than 24 hours after production, usually the day after the filtration experiment; if left for longer than this period there would be significant bacteriological breakdown of organic material, thereby affecting the results. Samples of the algal ration used in the filtration rate experiment were also taken. These samples were filtered through pre-weighed, ashed Whatman™ GF/C filters. The samples were then dried (at 70°C) and ashed (at 450°C).

$$\text{Absorption Efficiency (e)} = \frac{F-E}{(1-E)F}$$

where F = Ash free dry weight : dry weight of food (seston)

E = Ash free dry weight : dry weight of faeces

RESPIRATION RATE

Respiration rate was measured as described by Hawkins and Hutchinson (1990) in flow-through respirometers supplied with water of appropriate temperature and salinity from the micro-processor controlled system. Winkler titration was not used to measure oxygen depletion in the water as it was the relative change in oxygen concentration, and not the absolute concentration required, and it was felt that oxygen meters would provide accurate enough results. To measure the respiration rate of each animal, water flow was shut off and Schott-Geräte™ oxygen meters and probes were used to measure the linear decrease in oxygen concentration in each chamber. The water in each respirometer

was stirred with a magnetic stirrer and maintained at a constant temperature using a water bath. Values were corrected against a control respirometer, with no animal, and calculated from the equation :

$$\begin{aligned} \text{Oxygen Consumption Rate } (V_{O_2} \text{ ml } O_2 \text{ l}^{-1}) \\ = (Ct_0 - Ct_1) \times (V_c - V_a) \times \frac{60}{t_1 - t_0} \end{aligned}$$

where V_c = volume of chamber (0.85 l)

V_a = volume of animal (in litres, measured by displacement of water)

t_0 and t_1 are time in minutes

Ct_0 = O_2 mg l⁻¹ at t_0

Ct_1 = O_2 mg l⁻¹ at t_1

60 = time between each reading

(N.B. the O_2 values were x 0.7197 to convert from mg l⁻¹ to ml l⁻¹)

EXCRETION RATE

Nitrogenous excretion was measured separately from respiration rates, using the method described by Solorzano (1969). Animals were maintained in separate vessels, in a volume of 250 ml of appropriate temperature/salinity sea water, for a period of 2 hours. Experimental results were corrected against a control of ambient sea water. A 10 ml seawater sample was taken from each vessel; these aliquots were treated in sequence with 0.4 ml phenol solution (10 mg phenol in 100 ml 100% ethanol), 0.4 ml sodium nitroprusside solution (0.5g in 100 ml distilled water), and 1.0 ml of oxidising solution (table 2.01). Each sample was thoroughly mixed between each addition. The final samples were then covered with aluminium foil and incubated in the dark, at room temperature. Readings were made using a CECIL™ CE 292 spectrophotometer at 640 nm, with a 10 mm light path, after 2 hours and before 24 hours. The concentration of ammonia

excreted was calculated from the equation :

$$\begin{aligned} \text{Ammonia Excretion Rate } (\mu\text{g NH}_4\text{-N h}^{-1}) \\ = \frac{(B_a - B_c) \times 14}{1000/V} \times \frac{1}{t} \end{aligned}$$

where V = water volume in chamber (0.25 l)

B_c = $\text{NH}_4\text{-N}$ in control chamber (in μM)

B_a = $\text{NH}_4\text{-N}$ in animal chamber (in μM)

t = time in hours (2 h)

14 & 1000 = conversion factors from $\mu\text{M NH}_4\text{-N}$ to $\mu\text{g NH}_4\text{-N}$.

Ammonia concentrations were calculated from a regression obtained from a series of ammonia standards. These were prepared using a deionized-distilled water (DW) blank and standard stock solution of 1 mM NH_4Cl in the following concentrations :

1 μM (10 μl stock in 10 ml DW)

5 μM (50 μl stock in 10 ml DW)

10 μM (100 μl stock in 10 ml DW)

20 μM (200 μl stock in 10 ml DW)

40 μM (400 μl stock in 10 ml DW)

These standards were treated in the same way as the samples.

OXIDISING SOLUTION	
Sodium Hypochlorite (min 5% Cl)	5 ml
Alkaline Solution	20 ml
ALKALINE SOLUTION	
triSodium Citrate	100 g
Sodium Hydroxide	5 g
Distilled Water	500 ml

Table 2.01 Oxidising solution used in Solorzano method for ammonia detection.

SCOPE FOR GROWTH

The physiological measurements described can be converted a Scope for Growth (SFG) energy index. This index can be calculated from the equation :

$$P \text{ (J h}^{-1}\text{)} = A - (R + U)$$

where 1. Scope for Growth = $P \text{ (J h}^{-1}\text{)}$

2. Energy consumed (C) :

$$C = \text{clearance rate (l h}^{-1}\text{)} \times \text{energy content of algae (J l}^{-1}\text{)}$$

[where algal cell numbers were converted from cells l⁻¹ to mg l⁻¹ and thence to J l⁻¹. The calorific value of the algae was taken as 23.5 J mg⁻¹ (Widdows *et al.*, 1979), which has been cited as a typical energy value for most

organisms and food materials (Slobodkin and Richman, 1961), and was the value used in a previous study of *Ostrea edulis* (Hutchinson and Hawkins, 1992) allowing comparisons between the two species.]

3. Energy absorbed from the seston (A) :

$$A = C (J h^{-1}) \times e$$

[where e = absorption efficiency]

4. Energy respired (R) :

$$R = Vo_2 (ml O_2 h^{-1}) \times 20.33$$

[N.B. the oxygen consumption is converted to Joules by multiplying ml O₂ h⁻¹ by 20.33]

5. Energy excreted (U) :

$$U = NH_4 (\mu g NH_4-N h^{-1}) \times 0.0249$$

[N.B. the ammonia excretion is converted to Joules by multiplying $\mu g NH_4-N h^{-1}$ by 0.0249]

BODY CONDITION INDEX

A body condition index (BCI) was calculated for all animals as a simple method for determining physiological condition. Whole animal and shell volumes were measured by displacement of water, and soft animal tissue was dried in a 70°C oven. The considerable variation of water content in wet tissues leads to apparent fluctuations in the BCI, so dry tissue weight was used in the index described by Walne (1970) :

$$BCI = \frac{\text{Dry Tissue Weight (g)}}{\text{Shell Cavity Volume (ml)}} \times 1000$$

2.2.3 Statistical Analysis of Results

The results of laboratory experiments were expressed as mean values \pm standard error, unless otherwise stated. The number of observations at each datum point was 12. Where appropriate data were analysed using a t-test (or a Mann-Whitney test as a non-parametric alternative), for comparing two conditions, or a standard ANOVA (or a Kruskal-Wallis test as a non-parametric alternative) for comparing more than two conditions. Pearson product-moment correlations were performed to measure the association between variables; this test assumed the data were normally distributed and did not assign dependent and independent variables. Comparisons were made between each combination of the five temperatures and six salinities.

2.3 RESULTS

2.3.1 Scope for Growth

Animals were fed with a particulate concentration of approximately 25,000 cells per cm^3 and the filtration rates measured for this concentration are indicated in figure 2.01. At 5°C there was no significant difference between filtration rates at low or high salinities ($P > 0.05$). The change in filtration rate which occurred between 10°C and 15°C was significant at high salinity ($P < 0.001$), with differences in filtration rate in more dilute water proving non-significant ($P > 0.05$). Significant differences in filtration rates across all salinities occurred between 5° & 10°C , 10° & 15°C , and 15° & 20°C ($P < 0.001$ in all cases). However, at high salinity no significant differences in filtration rates occur between 20° & 25°C ($P > 0.05$). When the filtration rate at different salinities was compared the only significant differences occurred between 22‰ and 28‰ ($P < 0.025$); the other differences were not significant ($P > 0.05$). The only significant effect of salinity on absorption efficiency (figure 2.02a) occurred between 19‰ and 25‰ ($P < 0.025$). The temperature had no significant effect on the absorption efficiency (figure 2.02b) of the oysters ($P > 0.05$). The combination of temperature and salinity had a highly significant effect at 16‰, between 10°C and 15°C ($P < 0.001$), but differences between other points were not significant ($P > 0.05$).

Figure 2.03 shows a marked separation in the respiration rates at high temperatures compared with low temperatures; the differences between 15° & 20°C , and 20° & 25°C were both highly significant ($P < 0.001$). The differences in respiration between 5° , 10° and 15°C were all non-significant ($P > 0.05$). In figure 2.03b, it is shown that up to 15°C the respiration rates remain constant, but above this temperature they increase linearly. Salinity (figure 2.03a) appeared to have little effect on respiration, with the only significant differences occurring between 22‰ & 33‰ ($P < 0.01$), and between 22‰ & 28‰ ($P < 0.025$).

Mean excretion rates (figure 2.04) showed a general increase with temperature, with significant differences occurring between 10° & 15°C , and 15° & 20°C (both $P < 0.001$). These measurements proved to be the most variable of the physiological tests made, and frequently reflected the 'health' of the animals

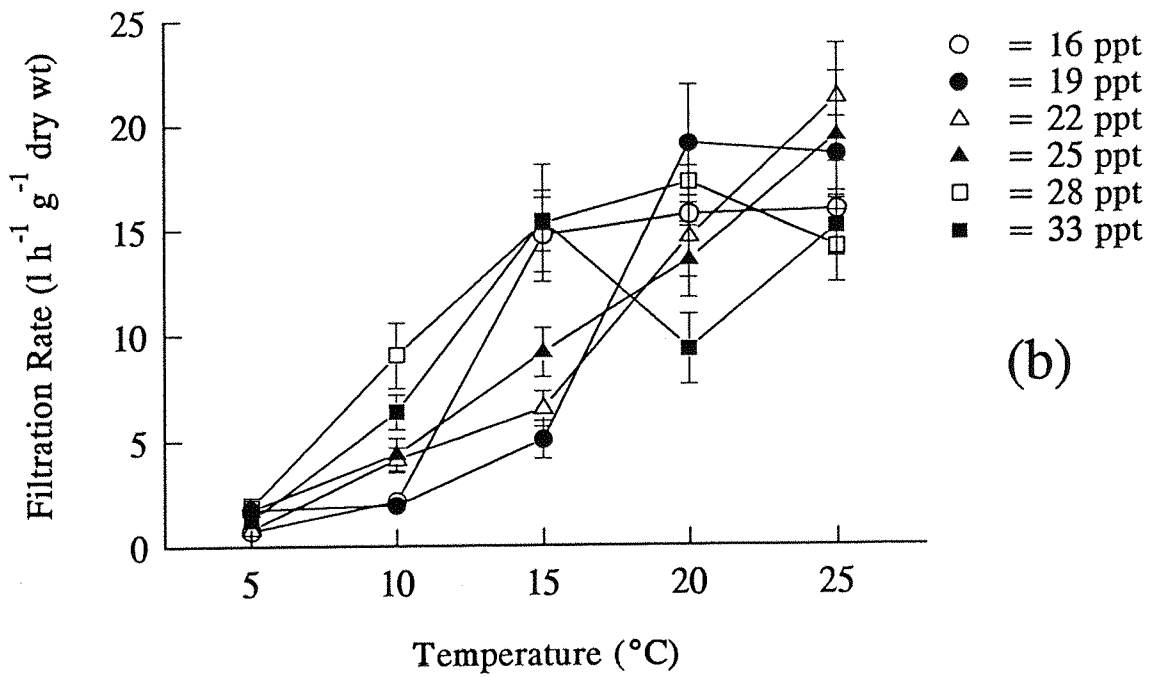
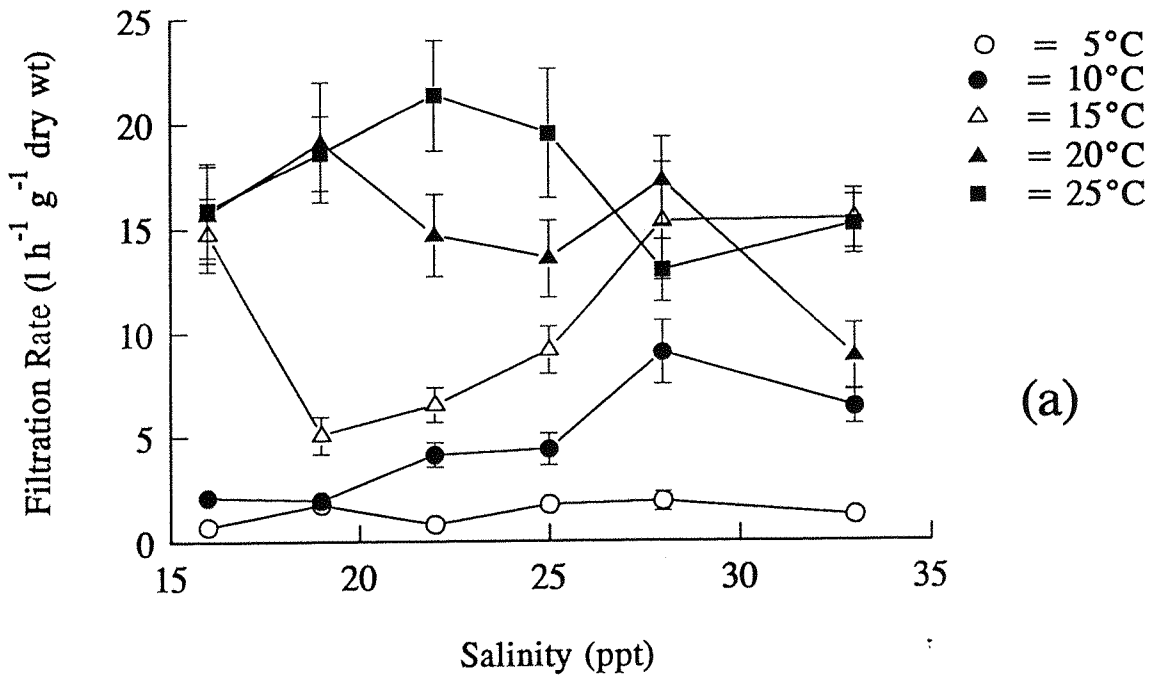


Figure 2.01 The effect of (a) salinity and (b) temperature on the filtration rate ($\text{l h}^{-1} \text{g}^{-1} \text{dry weight}$) of *Crassostrea gigas*. Mean \pm SE, $n = 12$ at each point.

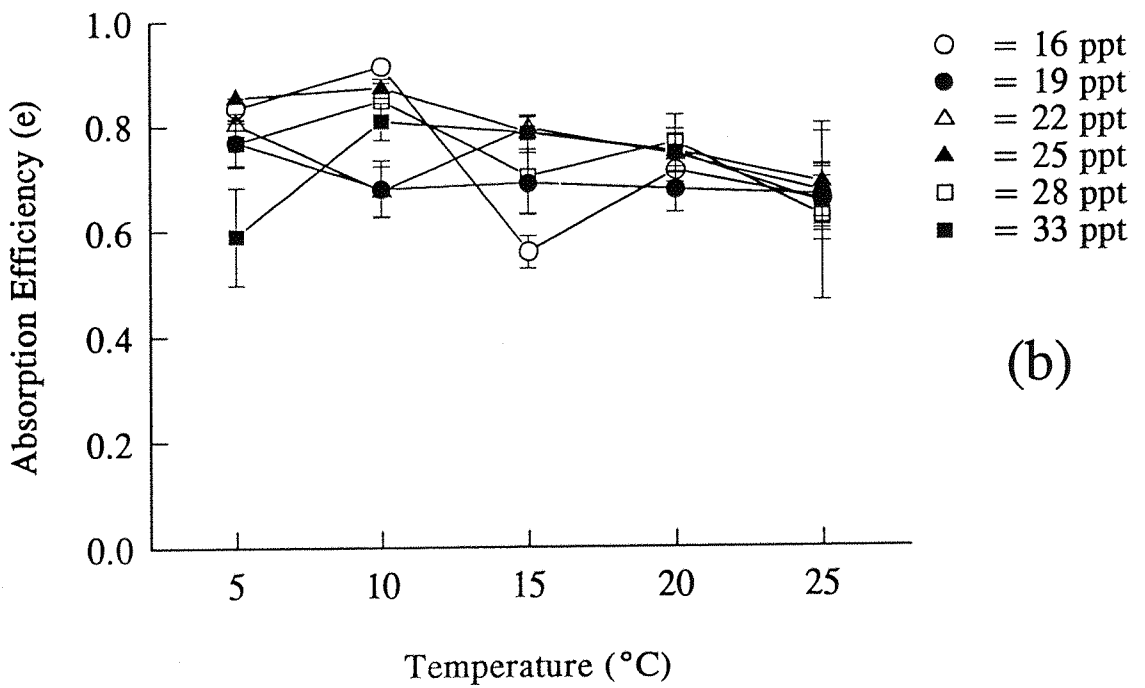
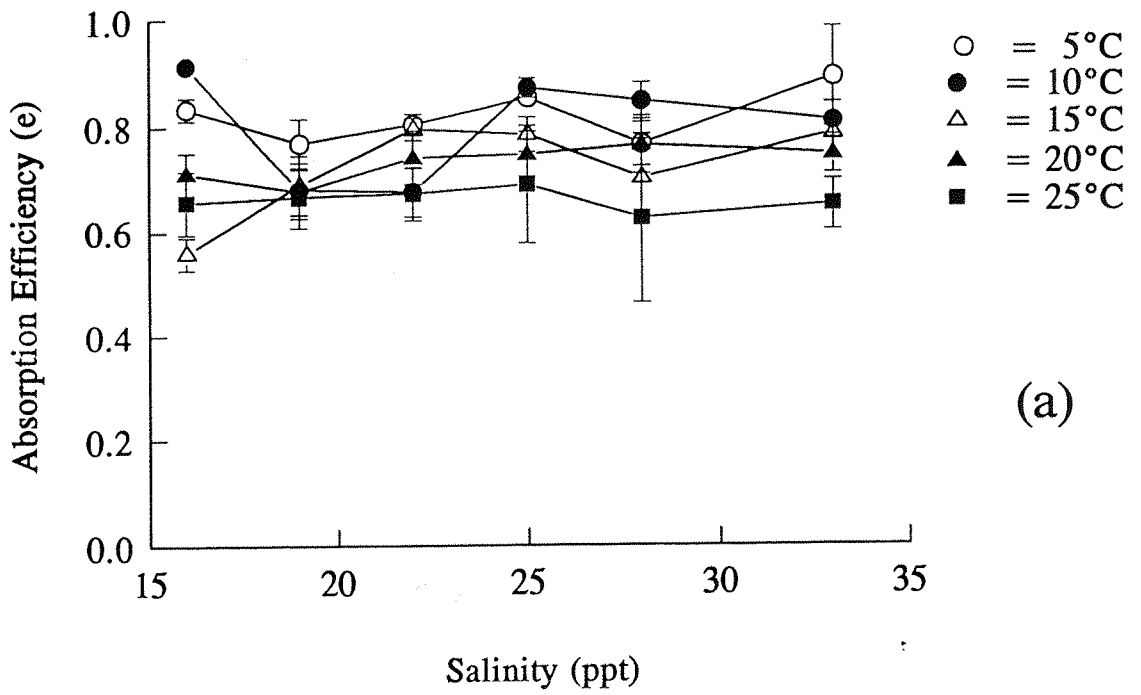


Figure 2.02 The effect of (a) salinity and (b) temperature on the absorption efficiency (e) of *Crassostrea gigas*. Mean \pm SE, n = 12 at each point.

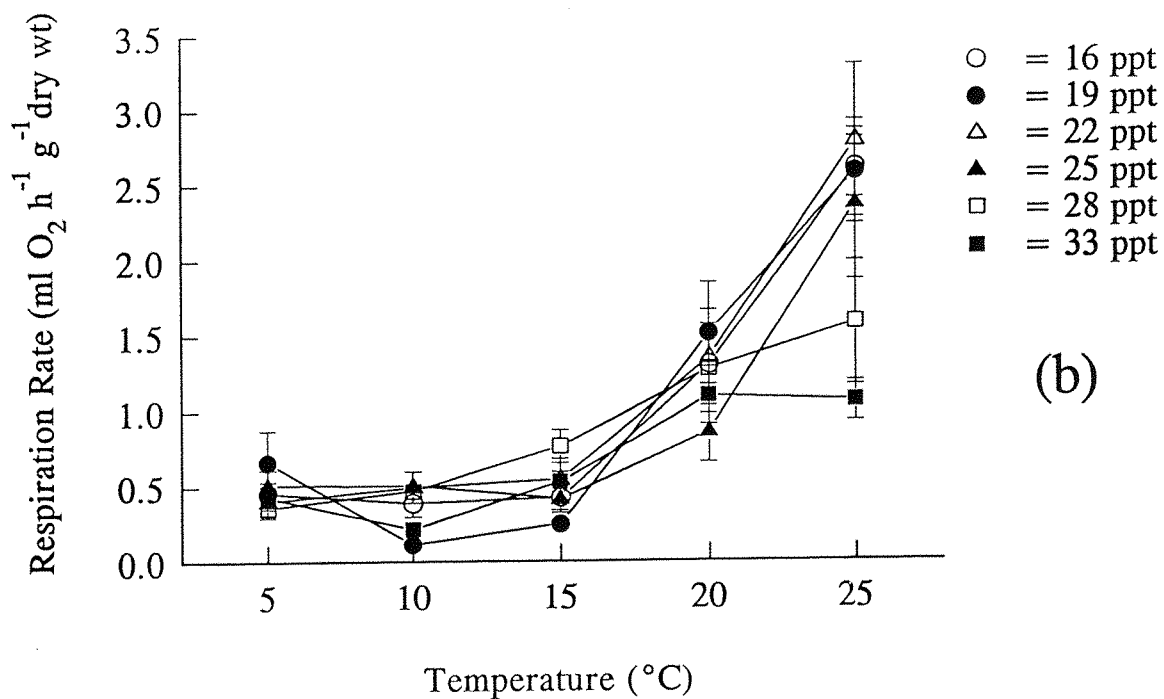
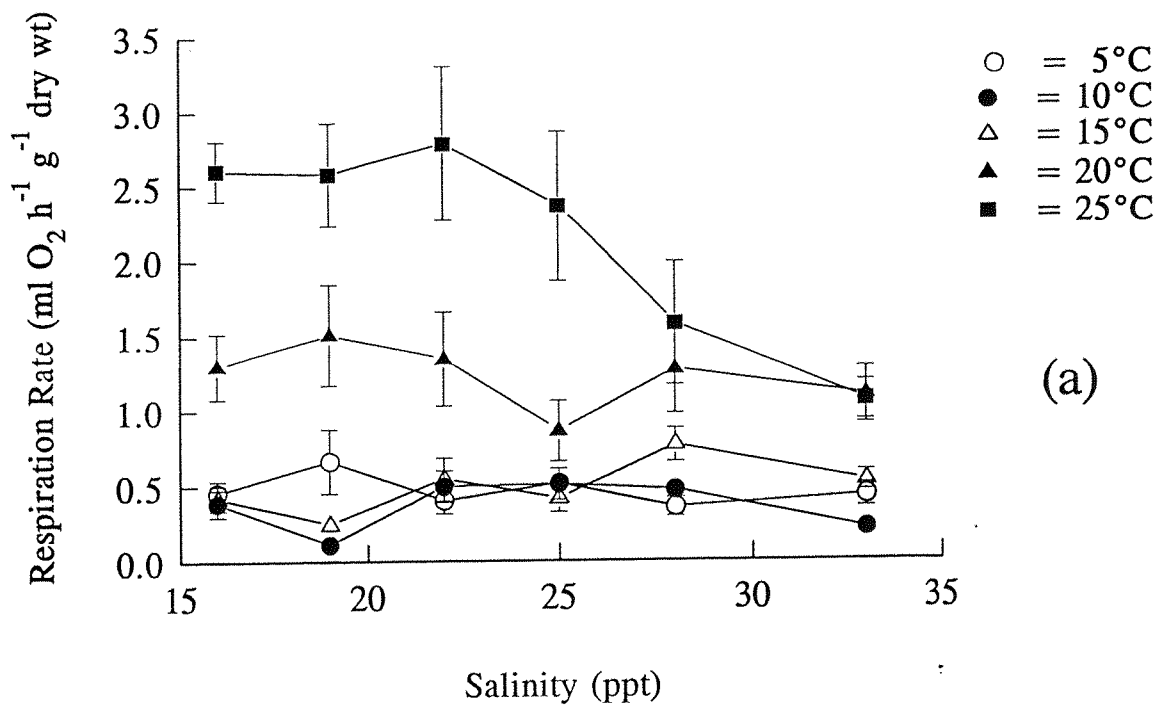


Figure 2.03 The effect of (a) salinity and (b) temperature on the respiration rate (ml O₂ h⁻¹ g⁻¹ dry weight) of *Crassostrea gigas*. Mean \pm SE, n = 12 at each point.

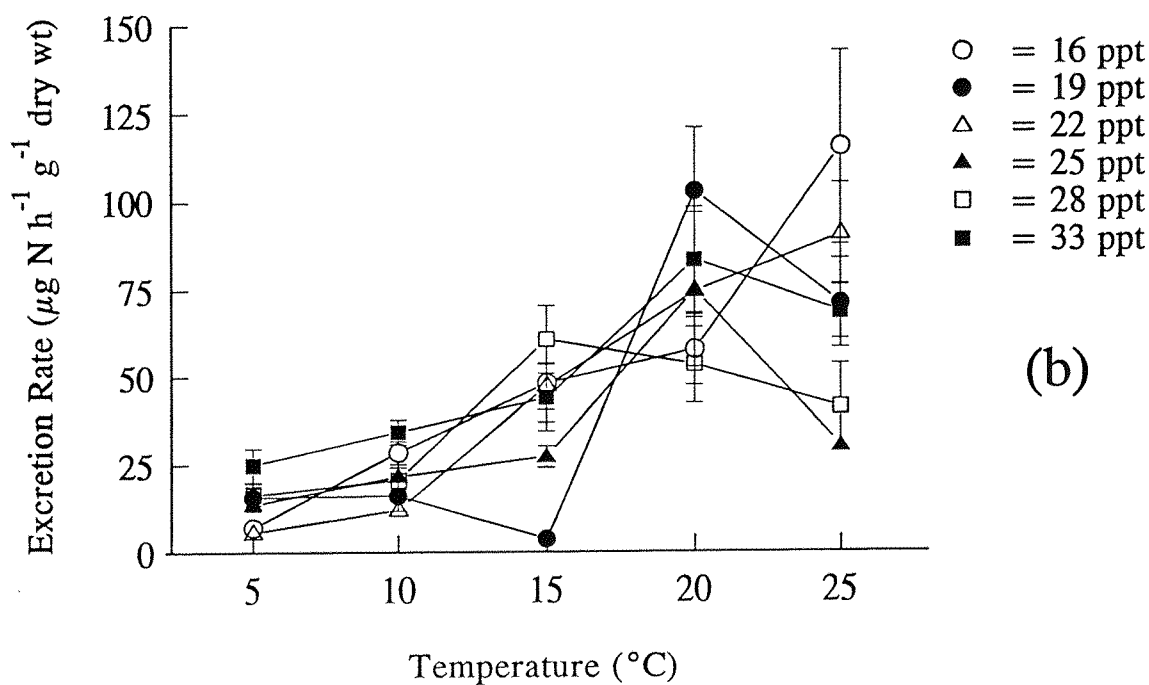
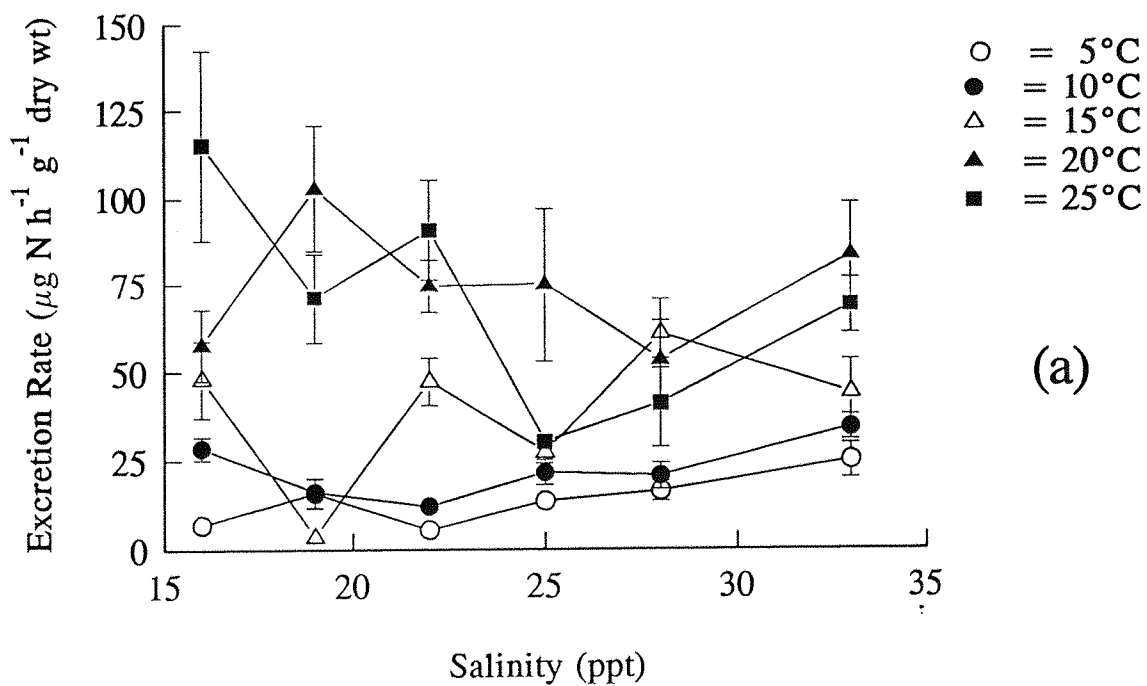


Figure 2.04 The effect of (a) salinity and (b) temperature on the excretion rate ($\mu\text{g N h}^{-1} \text{g}^{-1} \text{dry weight}$) of *Crassostrea gigas*. Mean \pm SE, $n = 12$ at each point.

(unpublished observations) as animals with a reduced body condition index showed higher nitrogenous output. There was little difference between excretion at temperatures above 20°C ($P > 0.05$). The differences in excretion rate were significant between low (16‰), mid (25‰) and high (33‰) salinity ($P < 0.001$), but no significant difference ($P > 0.05$) was found when a small salinity range was measured (for example 16‰ versus 19‰, or 25‰ versus 28‰).

Scope for growth (SFG) values have been calculated for only one value of particulate organic matter (1.0 mg l⁻¹). The pattern of values for this index (figure 2.05) was very similar to that obtained for the filtration rates (figure 2.01). A Pearson product-moment correlation gave a highly significant, positive correlation ($r = 0.948$; $P < 0.001$). The SFG values showed distinct separations between each temperature (figure 2.05a); significant differences occurred between 5° & 10°C ($P < 0.01$), 10° & 15°C ($P < 0.001$), and 15° & 20°C ($P < 0.01$). The change between 20°C and 25°C was not statistically significant ($P > 0.05$). The differences between each salinity, shown in figure 2.05b, proved to be non-significant except for a few specific points - between 10°C/16‰ & 15°C/16‰ ($P < 0.025$), 10°C/33‰ & 15°C/33‰ ($P < 0.001$), 15°C/33‰ & 20°C/33‰ ($P < 0.025$), and 25°C/16‰ & 25°C/33‰ ($P < 0.05$).

2.3.2 Body Condition Index

The body condition index (BCI) for the different temperature and salinity combinations is shown in figure 2.06. The differences in mean values for different temperatures were greater than would be expected by chance but pairwise multiple comparisons showed there were no statistically significant differences ($P > 0.05$). Certain individual points, when compared, proved to be significantly different. BCI at 20°C/33‰ & 25°C/33‰ were significantly different from all other salinities at these temperatures ($P < 0.05$) and from 16‰, 25‰ & 28‰ at the lower temperatures ($P < 0.05$). There was no significant difference in BCI with the 19-22‰ salinities at low temperature ($P > 0.05$) and there was no significant difference in the mean values at each salinity ($P > 0.05$). Figure 2.06a indicates the best BCI occurs at low temperatures (10-15°C) and either low (19-22‰) or

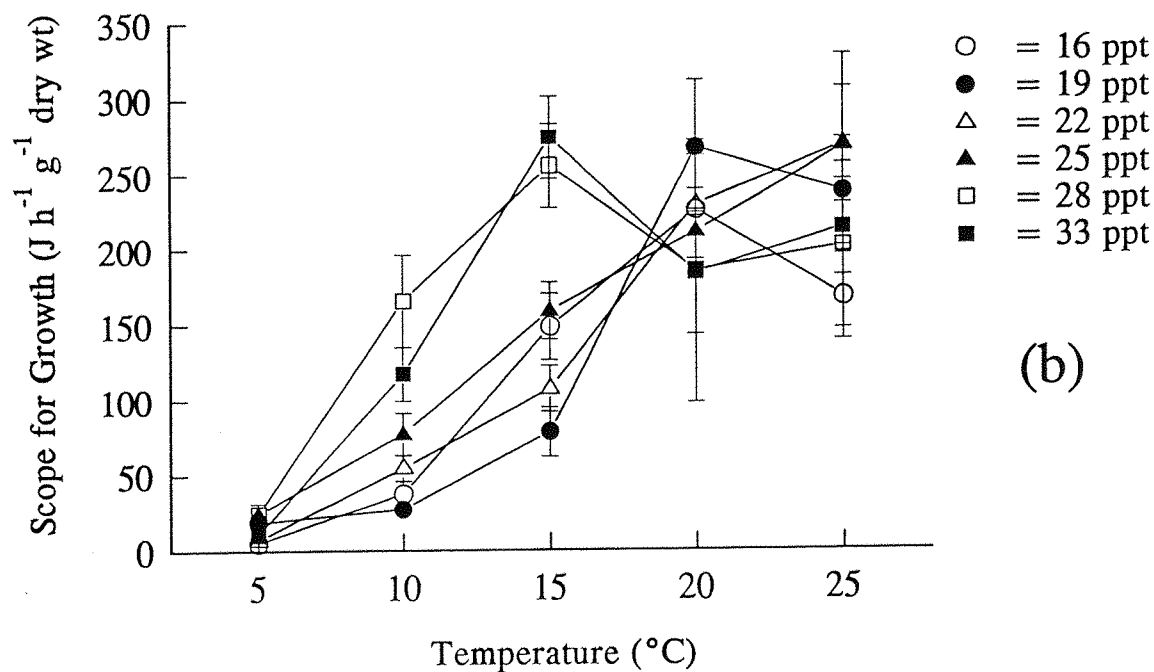
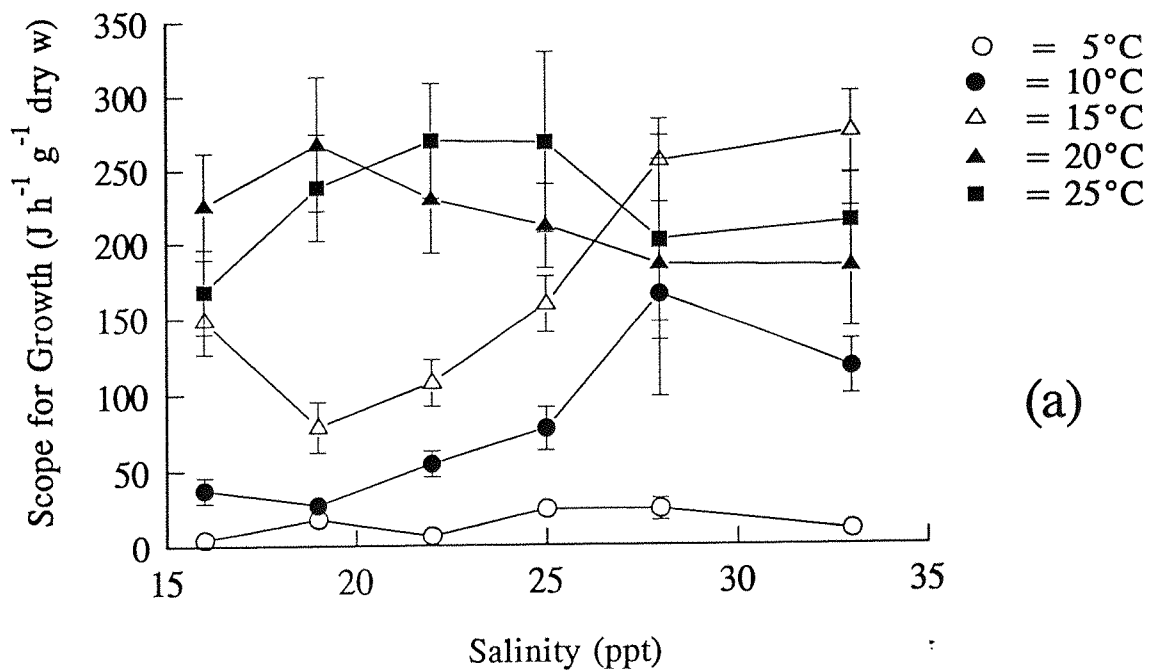


Figure 2.05 The effect of (a) salinity and (b) temperature of the scope for growth ($\text{J h}^{-1} \text{g}^{-1} \text{dry weight}$) of *Crassostrea gigas*. Mean \pm SE, $n = 12$ at each point.

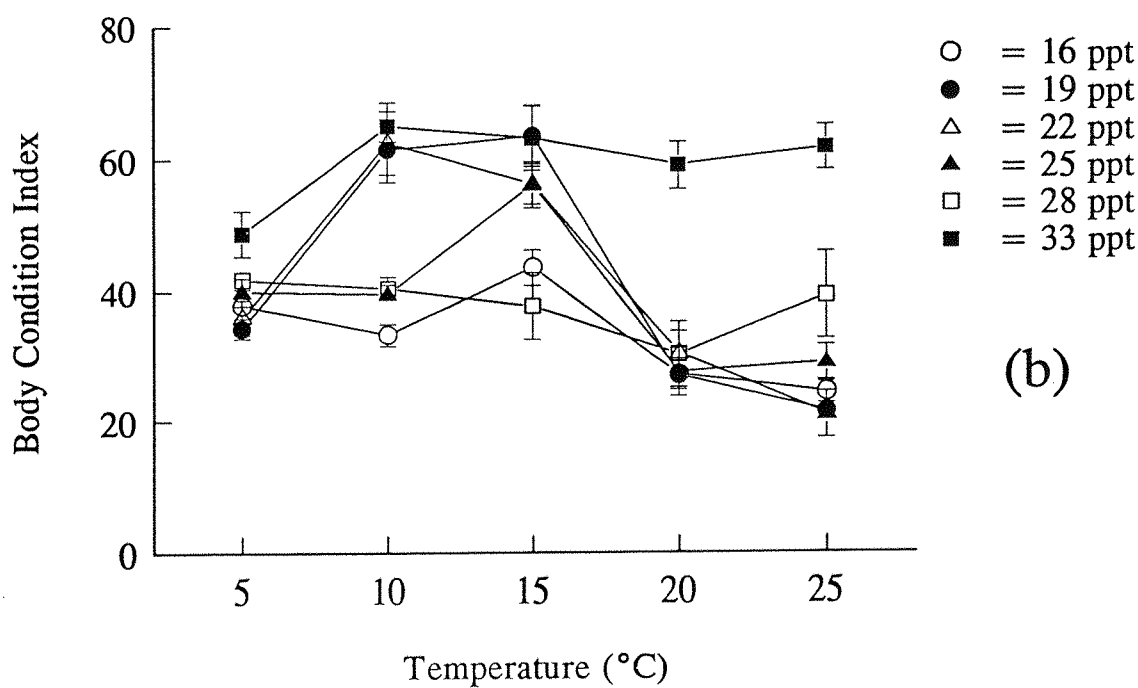
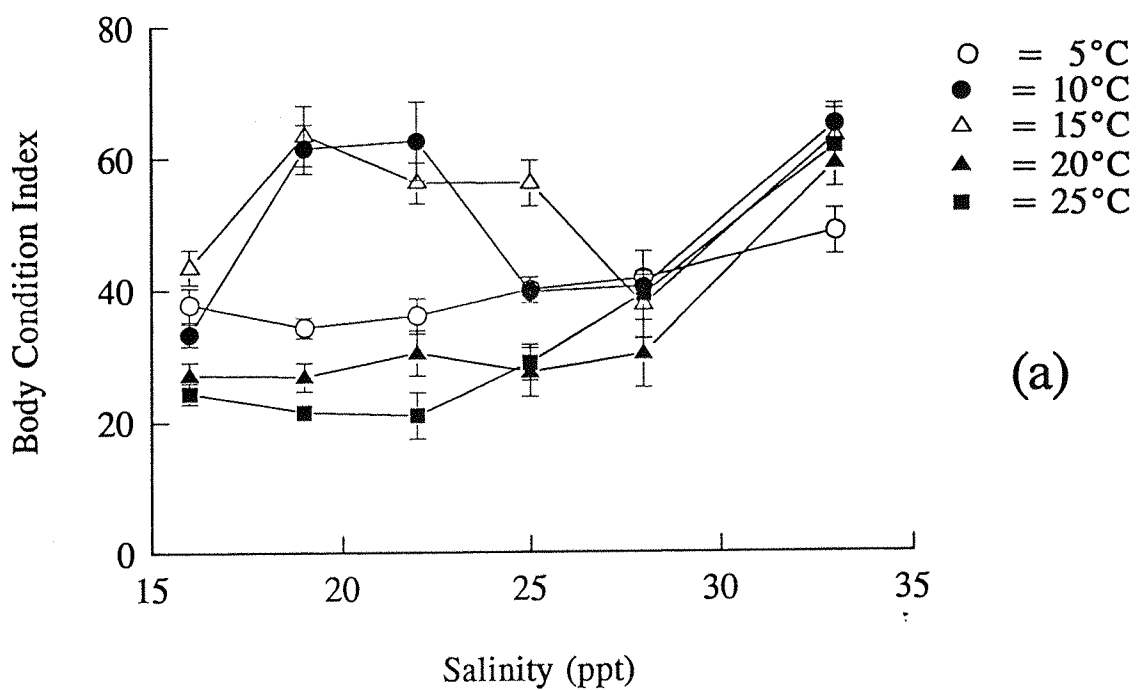


Figure 2.06 The effect of (a) salinity and (b) temperature on the body condition index of *Crassostrea gigas*. Mean \pm SE, $n = 12$ at each point.

high (33‰) salinity. Most temperatures showed their highest BCI at 33‰ and these results indicated a preferred range of environment which is different from that measured for the SFG index. This was supported by a highly significant, slightly negative correlation ($r = -0.245$; $P < 0.001$). A similar relationship occurred for the SFG and BCI measurements of the three populations of *Ostrea edulis* discussed in the next chapter.

2.3.3 Energy Budgets

The different parameters of SFG can be expressed as energetic equivalents and their relationship with temperature is shown in figure 2.07. A logarithmic scale has been used here because the change in energy of SFG is made clearer. At 5°C (figure 2.07a) very little energy was consumed and absorbed. The variation with respect to salinity shows there is minimal energy for SFG at 16‰ (4.71 J h⁻¹) and maximum energy at 25-28‰ (24.35 J h⁻¹). A 10°C rise in temperature (figure 2.07b) showed significantly more energy was assimilated ($P < 0.05$) but with no significant change in the amount of energy lost through respiration and nitrogenous excretion ($P > 0.05$). This led to a significant increase in energy available for somatic growth and gametogenesis ($P < 0.05$). A further increase to 25°C (figure 2.07c) gave a non-significant ($P > 0.05$) change in the energy assimilated, but a significant increase in the energy lost through respiration and excretion ($P < 0.05$). These changes mean SFG increases significantly between 5°C and 15°C ($P < 0.05$) but not between 15°C and 25°C ($P > 0.05$).

Salinity extremes did not have the same dramatic affect on SFG as temperature did (figure 2.08). The effect of the temperature gradient was again seen at 16‰ with a large proportion of SFG occurring at mid-temperature (15°C). As the salinity increased to 25‰ and to 33‰ there was little change in the animals' energy partitioning. This small change was reflected in the absence of statistically significant differences between any of the parameters ($P > 0.05$ for all comparisons).

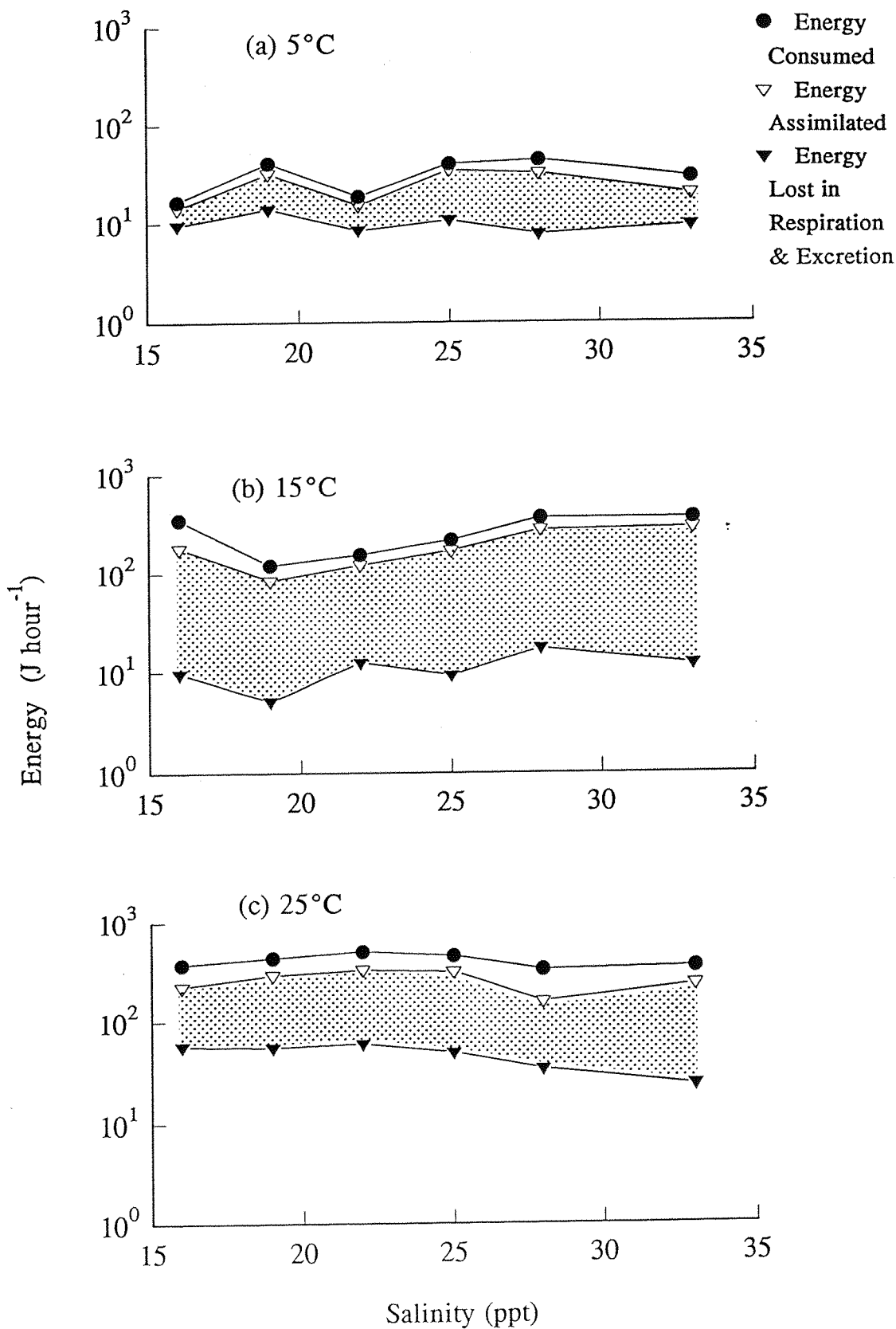


Figure 2.07 The effect of salinity, at three different temperatures, on scope for growth (shaded area) and components of the energy equation in *Crassostrea gigas*. Mean values, $n = 12$ at each point.

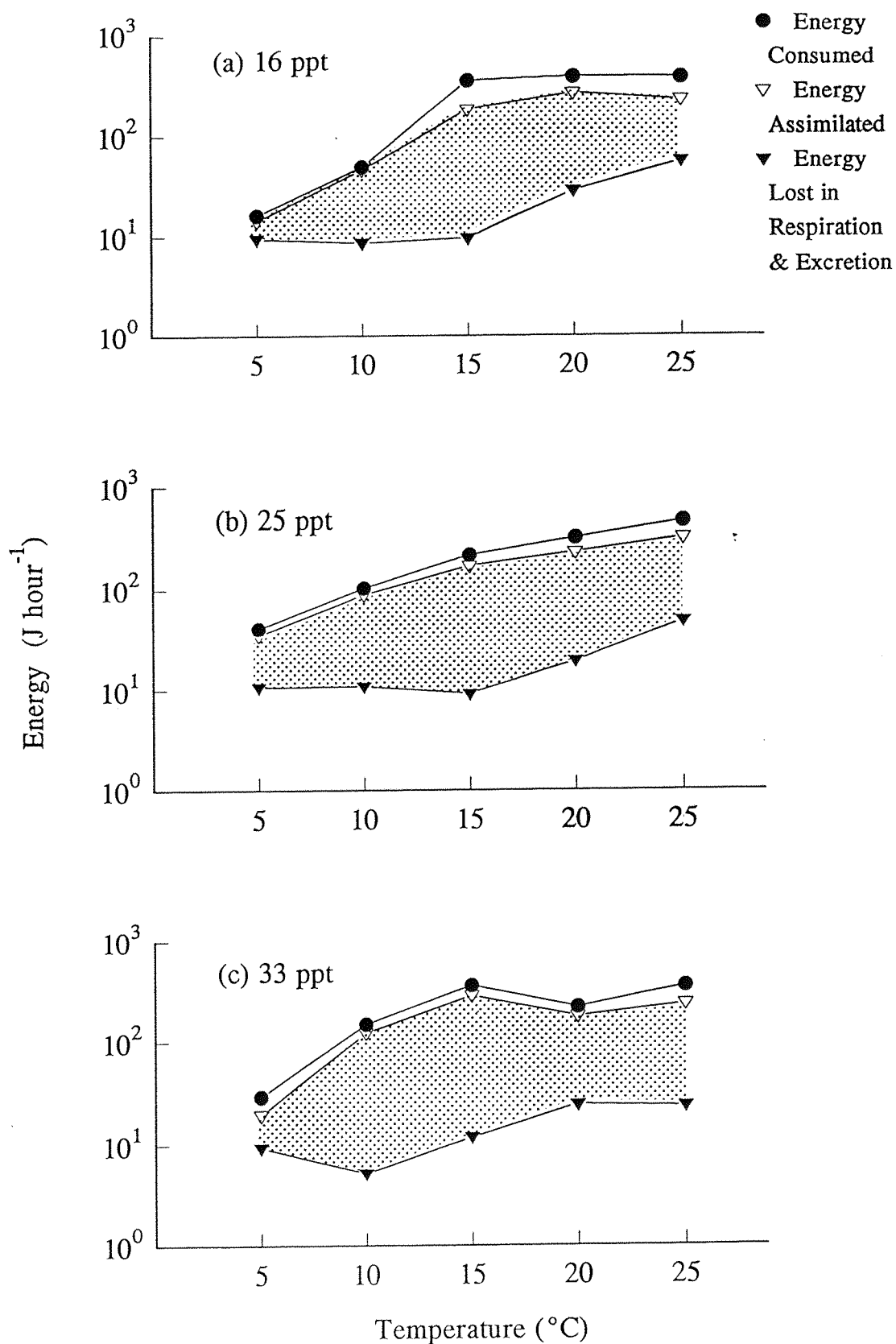


Figure 2.08 The effect of temperature, at three different salinities, on the scope for growth (shaded area) and components of the energy equation, in *Crassostrea gigas*. Mean values, $n = 12$ at each point.

2.3.4 Energy Partitioning

The energy budget for *Crassostrea gigas* at 15°C/33‰ was divided up proportionally and is shown in figure 2.09. The conditions of 15°C temperature and 33‰ salinity were selected as these represented mean conditions for both the Solent and the department aquarium, allowing better comparisons to be made between *C. gigas* and the Solent population of *Ostrea edulis*. The majority of energy was directed into somatic growth or gametogenesis; this was the same in energy budgets for populations of *O. edulis* and *Tapes philippinarum* which are discussed in the next chapter (sections 3.4.3 and 3.4.6 respectively). The proportion of energy for SFG in the Pacific oyster was not significantly different from these other species ($P > 0.05$) with the exception of the Loch Sween *O. edulis* which had significantly less energy for SFG than the others ($P < 0.05$).

One-fifth of the energy consumed by *C. gigas* was lost as faeces and pseudofaeces; the remaining assimilated energy was utilised in respiration and ammonia excretion. Construction of energy budgets for this and the other study species, under the same environmental conditions, showed many differences and similarities in the fate of consumed energy; these will be analysed and discussed more fully in section 3.4.3.

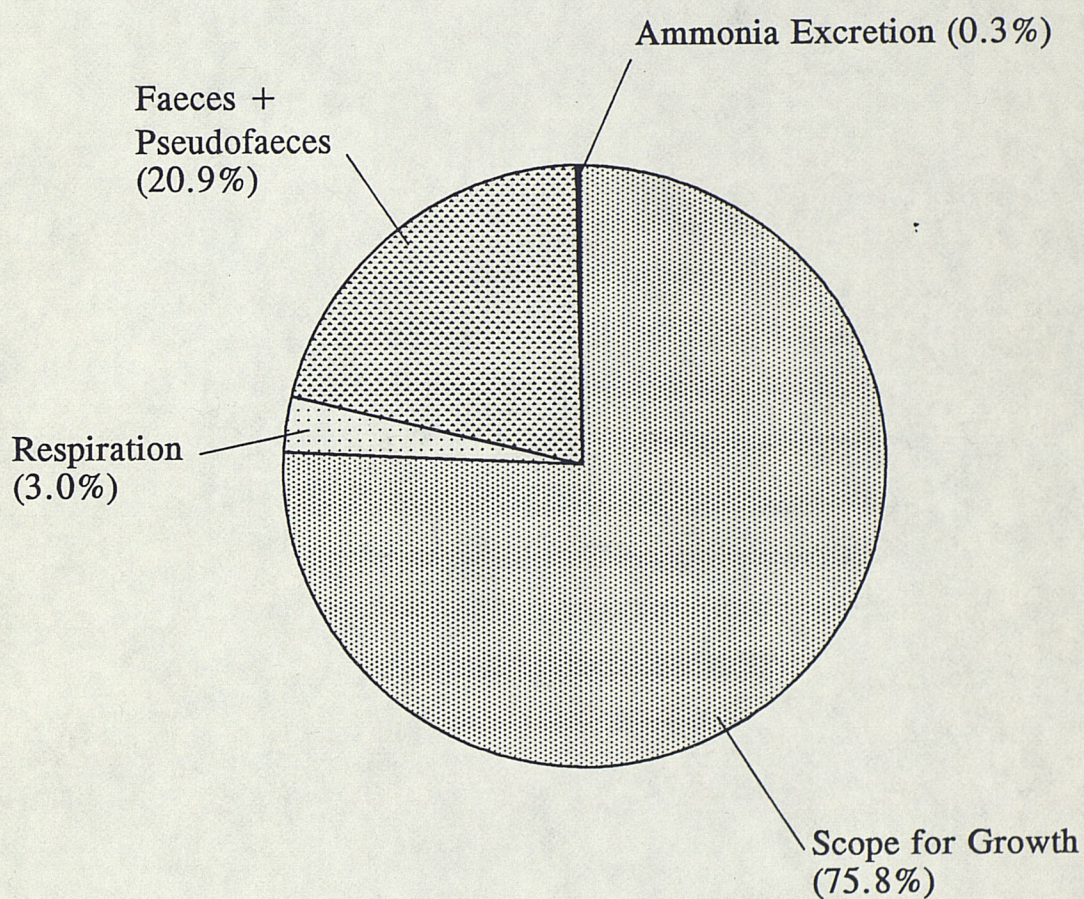


Figure 2.09 Partitioning of ingested energy in *Crassostrea gigas* at 15°C and 33‰. Mean values, n = 12.

2.4 DISCUSSION

2.4.1 Scope For Growth

FILTRATION RATE

Bivalve growth in nature is clearly a function of several environmental variables (Brown, 1988). Knowledge of growth and mortality rates are useful in economic forecasts for oyster culture, estimating the numbers of animals of marketable size per area (and thus the cost/profit margins), and also for the control of recruitment and harvesting of oysters to prevent overfishing and the failure of the harvest. Growth studies generally involve laboratory or field experiments which either manipulate conditions or monitor natural variations of variables. From the physiological parameters measured in this study, the component with the most significant effect on scope for growth (SFG) was the filtration rate (figure 2.05 compared with 2.01). This finding was consistent with the findings of Barber *et al.* (1991) and Hutchinson and Hawkins (1992). The highest rates of filtration occurred at high temperatures (20-25°C) but at low to mid salinity (19-25‰). As the salinity increased the filtration rate dropped. This may reflect that in the wild *Crassostrea gigas* is a warm water, estuarine species rather than a fully marine species. Fisher and Newell (1986) showed in *Crassostrea virginica* that protein and carbohydrate levels increased at higher temperatures, indicating an increased intake and some storage of food. However, they also showed the effects of salinity and temperature, alone and in combination, can vary with season and between study sites of selection. Similar differences were demonstrated in a study of *C. gigas* (Brown, 1988) with different site specific growth related to salinity and food supply variation, and monthly growth rates more affected by size and temperature. Mean monthly salinity at the low growth sites was frequently less than 20‰, whilst higher salinities were recorded in medium and high growth groups. Quayle (1969) and Bernard (1983) both stated that somatic growth occurs in *C. gigas* between 16‰ and 31‰, with the optimal conditions between 20‰ and 25‰, as demonstrated in the present study. It is possible that low salinity levels are more likely to be limiting to growth since *C. gigas* has been successfully cultivated in hyper-saline ponds at 40‰ (King, 1977).

The differences in filtration rate observed above 20°C were not significant

suggesting that this is its optimal temperature range. At 5°C the filtration rate was minimal as the animals ingested enough food to maintain their basic metabolic levels. These results showed a similar trend to *Ostrea edulis* but had a much wider range of rates which will be discussed more fully in section 3.4.4. High filtration rates are the major factor causing high growth rates recorded in *Crassostrea gigas* (Walne, 1979) and similarly give rise to the high SFG values recorded in this study.

The animals used in this study were of the same age and size range for each group, but in general the filtration rate will increase proportionally with body size and will decrease with increasing food concentration (Winter *et al.*, 1984). Food for the oysters consists of particulate organic matter, detritus and bacteria, with 25% of seston consisting of food during peak annual periods (Brown, 1988). The consideration of food quality and quantity, as well as oyster size, are very important for determining the true nature of environmental influence on filtration rate. This study used the same concentration and calorific content of algae, as well as similar flow rates through experimental tanks, for all the filtration rates measured. However, in the natural environment the oysters need some ability to regulate the ingestion rate in relation to the food concentration. Many bivalves have different regulatory mechanisms which vary with long term seasonal changes, as well as with short term effects such as increased suspended matter. Changes in filtration rate in *Mytilus edulis* and pseudofaecal production in *Cerastoderma edule* have been demonstrated as a short term response, to maintain maximum levels of energy gain, whereas long term adaptations include changing digestive tubule size and longer periods of food retention in the gut (Navarro and Iglesias, 1993). Much of the data relating to long and short term adaptations of filtration rate and feeding behaviour has been reviewed by Bayne (1993) and Navarro and Iglesias (1993) and so is not discussed here. Utting (1988) noted that suspended matter affects growth quality in *Mercenaria mercenaria*, which was reduced by 16% at a sediment concentration of 44 mg per litre, compared with normal levels. The composition of the food supply is also important in determining filtration and growth rates. Particulate organic matter (POM) is especially important for high growth rates and high SFG is achieved where there is a high POM : TPM (total

particulate matter) ratio (Brown, 1988). This usually coincides with peak temperatures. At sites of low growth the ratio is reduced. Widdows *et al.* (1979) noted that a high proportion of POM is as important as a high absolute value of POM, because increased PIM (particulate inorganic matter) will dilute the ingestion rate. Wilson (1987) also showed that in *O. edulis* and *Pecten maximus* culture areas with the highest particulate organic carbon had the highest growth rate. Shumway and Koehn (1982) indicated that *C. virginica* shows highest SFG at 0.54 g algal dry weight per litre.

Changes in the natural environment can lead to negative effects on oysters. In 1950 large phosphate inputs into Great South Bay, New York caused an excessive bloom of a *Chlorella*-like alga (Wallace, 1966). This increase in cell numbers clogged the gill pumping mechanism of oysters resulting in starvation of the animals. Knowledge of effects such as eutrophication are vitally important in the successful management of the oyster fishing industry.

ABSORPTION EFFICIENCY

The small range of absorption efficiency (60-90%; figure 2.02) and lack of statistical differences measured showed that the animals were little affected by temperature and salinity. Slightly higher efficiencies were obtained for the lower temperatures (figure 2.02b) and probably resulted from lower metabolic rates causing food to spend more time in the gut. Assimilation rates have been shown to be independent of food concentration and body size in *Ostrea chilensis* (Winter *et al.*, 1984). Under stressful conditions, when an oyster may keep its valves tight shut for long periods of time, reduced assimilation may be seen and, together with the additional metabolic cost of anaerobic maintenance, can result in lack of growth (Brown, 1988). Under the conditions employed for this study no oysters spent prolonged periods closed.

No specific method was employed to determine pseudofaeces production. However, it is well documented that this process only occurs above a threshold value of suspended particulate matter (Newell *et al.*, 1989; Jørgensen, 1990; Rice and Pechenik, 1992; van Haren and Kooijman, 1993). The particulate level of 1.0 mg l⁻¹ used for these assays is below the threshold value for pseudofaeces

production in *Crassostrea gigas* (Bernard, 1974) and also *Ostrea edulis* (Møhlenberg and Riisgård, 1978 as cited in Barillé *et al.*, 1993). Filtration and ingestion rates are very closely related for seston densities low enough to prevent pseudofaeces production, and under such densities all filtered material is ingested (van Haren and Kooijman, 1993). Under these circumstances the values calculated for absorption efficiencies are accurate reflections of the proportion of ingested energy available for somatic and reproductive purposes. Consequently, the scope for growth index calculated is not altered by misinterpretation of pseudofaeces production.

If it is assumed that there are no pseudofaeces produced then the regulation of food intake in *Crassostrea gigas* is as a result of change in filtration rate. This could explain the drop in filtration rate at 20°C/33‰ (where the algal ration used was relatively high compared with other experiments), and the high absorption efficiency at 15°C/16‰, when the algal ration was low. The small effects of temperature on absorption efficiency (figure 2.02) were comparable with the results of Widdows and Bayne (1971), Buxton *et al.* (1981), and Hutchinson and Hawkins (1992). This supports the evidence given by Wieser (1973) that many poikilothermic invertebrates can regulate their metabolism by acclimation, even when they are under thermal stress.

RESPIRATION RATE

In *Ostrea edulis* the respiration rate was shown to increase linearly between 10°C and 25°C, but remain at constant levels below 10°C (Hutchinson and Hawkins, 1992). This difference was ascribed to a change between 'winter' and 'summer' physiological states, a phenomenon also described in *Crassostrea virginica* (Fisher, 1988b). In the present study the rate increased linearly between 15°C and 25°C, for salinities in the range 16‰ to 25‰, and remained constant below 15°C (figure 2.03). At high salinities the increase in respiration with increasing temperature was less pronounced. A switch in physiology at mid-salinities and 15°C would be consistent with a warm water, estuarine species such as *Crassostrea gigas*. The differences in rates between 15°, 20° and 25°C were all significant, but with no significant difference in rates below 15°C, giving

statistical support to a change occurring at 15°C. *O. edulis* gives the highest respiration rates at high temperatures / high salinities (see section 3.4.4) but this combination of environmental parameters reduces the respiration rate in *C. gigas*. This may reflect the estuarine physiology of *C. gigas* which at high salinities may be considered to be under stress. Rapid fluctuations in salinity are energetically costly, for example in maintaining osmoregulation, and ventilatory recovery may take several days for fluctuations greater than 10‰ (Bernard, 1983), although decreasing oxygen tensions resulting from lower respiration rates do not affect the viability, size and phagocytic activity of haemocytes (Alvarez *et al.*, 1992). The ventilation function in *C. gigas* has been shown to be greatly reduced below 8-10°C so under these conditions low availability of food is not limiting because of the reduced metabolic requirements (Bernard, 1983).

Shumway and Koehn (1982) showed that oxygen consumption of *C. virginica* is much higher at low salinity (7-11‰) than high salinity (28‰) and is more pronounced with increasing temperature (10-30°C). They stated that animals with a higher respiratory energy expenditure would have less energy for input into somatic growth and gametogenesis and would therefore have lower growth rates. They would also have less energy available to cope with metabolic costs of stress and become more susceptible to disease infection. Barber *et al.* (1991) have shown that *Crassostrea virginica*, resistant to the parasite *Haplosporidium nelsoni*, have higher respiration rates during the summer months than susceptible animals. Consideration must therefore be given to the likely increase in disease susceptibility as the respiration rate decreases with changing environmental conditions. Barber's study also showed that clearance rate was high during summer in resistant compared with susceptible oysters. Figure 2.01b shows a similar pattern to that displayed by respiration (figure 2.03b), with the maximum clearance rates at low-mid salinity, reducing with high salinity and emphasises the effect prevailing conditions can have on the disease resistance of an animal.

NITROGENOUS EXCRETION

Nitrogenous excretion in the form of ammonia showed a general increase proportional to temperature with significant differences occurring between low,

mid and high salinities (figure 2.04b). As with the respiration rates, maximum excretion rate occurred at low to mid salinity and was slightly higher in *O. edulis* (Hutchinson and Hawkins, 1992)(see also section 3.4.4). It has been shown in *C. virginica* that for animals at high temperatures there is an increase in protein content (Fisher and Newell, 1986). This increase leads to raised nitrogenous excretion as protein is turned over. Excretion may also be part of an osmoregulatory process and has been documented by several authors (as cited by Hutchinson and Hawkins, 1992). The general increase in rate of excretion starting between 10°C and 15°C, again reflected the 'winter/summer' physiology changeover point, with little activity occurring below this temperature. This physiological process is, like the others, influenced most heavily by temperature (figure 2.04b), with salinity (figure 2.04a) having little effect in either a positive or negative manner.

SCOPE FOR GROWTH

When all the components of scope for growth (SFG) are considered it is clear that the major influence on this index is that of filtration rate (figure 2.01 compared with figure 2.05). The animals at low to mid salinity (16‰-25‰) had a markedly lower SFG value at 15°C than did animals at high salinity (28‰-33‰). Above 15°C this changed with low salinities providing the highest levels of SFG. The changeover of behaviour may be an indicator of discrete ecotypes, i.e. physiological phenotypes which are able to function optimally under specific environmental conditions. This phenomenon is very difficult to explain concisely because of the multiplicity of influencing factors determining SFG. A variety of authors have suggested ideal conditions for the maximum growth of oysters, but very few discuss the influence of the different components. Krishnamoorthy *et al.* (1980) reported in *C. virginica* that meat weight is dependent on the age, sex, gamete production and spawning condition of the oyster, but fluctuations in weight are attributable to seasonal water temperature changes. Berrigan (1990) predicted that growth rates would be highest during periods when the water temperature was cooler since higher temperatures promote spawning and the use of energy, i.e. less energy is available for growth. Information taken directly from the field cannot

necessarily be directly applied to a different population because the data may not be representative of all populations. Even within the same area conditions can vary, as shown by the vagaries of the environment throughout Apalachicola Bay which strongly influenced rates of recovery in individual oyster reefs after hurricane damage (Berrigan, 1990).

Growth efficiencies are not expressed here as most figures recorded in the literature are for animals under natural conditions. Bivalves in the wild are frequently faced with less than optimal food quality, unlike those in artificial culture systems which, when fed with a mixed algal system demonstrate much more rapid growth (Rice and Pechenik, 1992). The animals used in the current study had relatively high growth efficiencies as the suspended particulate matter used was relatively low and consisted of totally labile material, ensuring a high degree of energy available for growth purposes.

This study of physiological responses over a matrix of environmental variables has not only determined optimal and detrimental combinations of variables, but has also allowed better predictions of what would occur in specific natural environments. It is also useful to know how oysters grow under different conditions to predict and avoid commercial problems, e.g. toxic bivalves - animals grown at high salinities are more prone to accumulate the toxic red alga *Gymnodinium breve* than animals at low salinity (Cummins *et al.*, 1971) and different species of bivalves show different levels of toxicity when taken from the same site. Bivalve growth in nature is clearly a function of several environmental variables, as stated by Brown (1988), and although predictive landings of species such as *Mya arenaria* or spatfall of *C. virginica* can be made from temperatures, salinity and precipitation (Ulanowicz *et al.*, 1982), food supply and quality, dissolved oxygen, pH and suspended sediments also have effects on SFG. For this study high SFG can be maintained with high temperatures/low salinities or low temperatures/high salinities, but not with other combinations; as the temperature increased (to the range more normally associated with tropical Pacific water) the normal estuarine salinity range gave optimal conditions for growth. Very low SFG at 5°C (figure 2.05a) demonstrated the basal metabolism is just enough to maintain animal tissues.

2.4.2 Body Condition Index

The body condition index (BCI) showed no significant pattern overall, although some differences between individual points were significant (figure 2.06). Under the conditions favourable for SFG there was a negative correlation with the observed BCI. When there was a high SFG the visceral mass was watery and did not fill the mantle cavity, resulting in low BCI; when SFG was low the body meat substantially filled the oyster shell cavity, giving a high BCI. It would appear that when SFG is high, shell is laid down and little or no energy is put into somatic or gametic production. High BCI is achieved when energy is directed mainly to gonad development, filling the mantle cavity, and less towards shell production. Orton (1928) observed a pause in shell growth during summer associated with spawning. Krishnamoorthy *et al.* (1980) noted that oyster meat increases between October and April, and wastes away between April and October as animals spawn. Males appear to lose weight for a longer period than females. There is also a significant correlation between meat weight and shell length. These workers recorded that shells grow actively in oysters until they reach a marketable size and that 'whole animal weight growth' is probably size related. Walne (1958) also argues that shell growth is continuous.

The sessile nature of oysters leads to chronic exposure to various stress inducing agents including pollutants (Couch, 1985). A study monitoring condition indices, based on the dry weight : cavity volume ratio, found significant variations between polluted and clean sites (Roper *et al.*, 1991). Oysters from the polluted site had thickened shells and chambering within the mantle cavity and seasonal variations were also observed with low condition between summer and winter, and high condition in spring and summer. A typical decrease in BCI resulting from spawning can be as much as 40-45% (Marcus *et al.*, 1989). When investigated further it was found that changes in BCI at the polluted site were caused by changing glycogen levels, but the clean site variations were caused by protein fluctuations. This suggests that the stress and metabolic cost of maintaining condition at the polluted site is more than the daily food intake provides, resulting in the utilisation of glycogen reserves. The use of protein at the clean site marks the switch from glycogen to protein metabolism during gonad proliferation.

Goldberg (1980) and Widdows (1985) have both observed reduction in condition index associated with stress.

The use of a gonadal index has been shown to vary inversely with the degree of parasitic infection by *Perkinsus marinus* in *C. virginica* (Gauthier *et al.*, 1990). During spawning there is a high energetic cost as gametes are released and the post spawning period, when gonad index is low, shows the oyster to be more susceptible to the parasite. In studying other parasites it was found that condition index was not correlated with the intensity of infection as with *P. marinus* although oysters in poor condition were usually infected with the sporozoan parasite *Nematopsis ostrearum*. Gametogenic development is correlated to temperature (Shpigel, 1989). In Israel the temperature never falls low enough to interrupt gametogenesis, leading to spawning throughout the year in *O. edulis*, but not in a native sub-tropical species *C. gigas*, which followed a normal spawning pattern. Unfortunately the author did not record BCI and levels of disease or parasitic infection but the study indicated the high adaptability of oysters to different conditions. The results for the present study also show the adaptability of *C. gigas* to a variety of environmental parameters - this is illustrated in figure 3.17 where a series of peaks are seen at high salinity for different temperatures and at low salinities for low temperature. Size also has a considerable effect on the growth of bivalves because the growth efficiency decreases with increasing age and size (Shumway and Koehn, 1982; Bayne and Newell, 1983). Reallocation of energy from somatic growth to gamete production occurs in giant scallops (MacDonald and Thompson, 1986) and the amount of energy utilised could be the final determinate of growth rate in mature bivalves.

The higher condition observed for *C. gigas* at all salinities except 33‰ for temperatures lower than 20°C (figure 2.06b) reflected the observation by Walne (1970) that, generally, oysters reared in cooler waters have high condition. Salinity (figure 2.06a) also showed some changes in BCI. At 10°C & 15°C there was high BCI between 19‰ & 25‰, which then decreased before increasing again at 33‰ - this compares very well with Quayle (1969) who gave two different optimum ranges for *C. gigas*, with the optimum for breeding being 20-25‰, and that for feeding being 25-35‰. King (1977) found a BCI of 135 at 40.9‰ and although

the highest body condition index found in this study was 65.16 the increased indices observed at 19-25‰ coincided with maximum levels of filtration rate. Developmental stages of the oysters were not recorded but it is likely that these high filtration rates provided added energy for gamete production. The high BCI at 33‰ occurred as respiration rates had decreased, i.e. there was less energy utilised in respiration and more energy available to somatic or gametogenic growth. The complicated nature of the interactions between physiological mechanisms means that body condition index in oysters, or other bivalves, needs to be used in conjunction with other indexation methods to draw meaningful conclusions as to the effect, or potential effect, of the environment on the animal. However, the index should not be dismissed as providing no information as it has clearly been demonstrated that useful data for monitoring or comparative work can be obtained.

2.4.3 Energy Budgets and Energy Partitioning

When SFG is studied in terms of the energy intake and expenditure the effects of temperature (figure 2.07) and salinity (figure 2.08) are more clearly seen. The small amount of energy available for somatic growth and reproduction at low temperature (figure 2.07a) reflected the animals' basal metabolism which assimilated just enough energy to maintain the body tissues. An increase in temperature (figure 2.07b) showed there is much more energy ingested and assimilated, without a significant change in energy lost. This makes more energy available for growth and for coping with stressful conditions which may arise, and consequently at this point the animals may be considered to be at their fittest or healthiest. A further 10°C rise in temperature did not provide the animal with any more energy, but it did increase the amount of energy lost through respiration and excretion. Although the decrease in SFG was not significant, resources would be more limited in times of stress, so animals can be considered to be less fit.

Salinity (figure 2.08) showed no significant effects on SFG in *C. gigas* between estuarine (16‰) and marine (33‰) salinities. This probably reflects the highly adaptive nature of this animal. Combinations of temperature and salinity

can be seen to have some effect on reducing or enhancing SFG from the non-linear patterns in the figures. Very often somatic growth of bivalves in the natural environment may be limited by factors other than the physical regime, for example food availability. A reduction in overall condition or energy stores may not be evident but the glycogen reserves can be affected (Utting, 1988). Under these conditions animals become stressed and are more susceptible to disease or parasitic infection. It therefore becomes important how an animal partitions the energy available to it. Figure 2.09 shows the fate of consumed energy for *C. gigas* at 15°C and 33‰. One-fifth of this energy is never available for growth because it is lost through faeces and pseudofaeces. The degree of this loss can vary depending on the physico-chemical conditions of the environment surrounding the oyster. High concentrations of bacteria in the water (100 cells per 100 ml water) can lead to oysters expending considerable energy in particle expulsion through pseudofaeces (Roper *et al.*, 1991); additional meat production then becomes very difficult. Figure 2.09 shows minimal assimilated energy is lost through respiration and nitrogenous excretion, which leads to 75% of the ingested energy being made available for SFG.

Few studies have been made of energy budgets and partitioning in oysters, but it has been shown that parasitic infection, for example by *Haplosporidium nelsoni* or *Perkinsus marinus* in *Crassostrea virginica*, causes a steady drain on the energy resources of the oyster (Newell, 1985; Barber *et al.*, 1988a; 1988b; Choi *et al.*, 1989; Hawkins *et al.*, 1993c). Heavy infections by *Perkinsus* result in the parasite consuming more energy than the oyster has available after meeting its own respiratory needs, i.e. the oyster has a negative energy budget (Choi *et al.*, 1989). This heavy, persistent drain on oyster energy only occurs under moderately heavy or heavy infections, but the effect at a given level of infection is smaller for smaller oysters. This is because respiration makes up a smaller fraction of the total energy budget in smaller oysters (White *et al.*, 1988a; 1988b). At 15°C/33‰, *C. gigas* has plenty of energy allocated for SFG and is much more likely to resist infection than if less energy were available. However, how that energy is utilised may also vary depending on environmental conditions. Oysters may increase in weight and volume but not in length or width at elevated temperatures. Under

these conditions the oyster requires a higher metabolic activity for existence, which leaves little energy left for shell growth (Burklew, 1971). Comparisons of different species under similar conditions will be discussed in sections 3.4.3 and 3.4.6, but the manner in which *C. gigas* partitions energy demonstrates how this species tolerates wide salinity changes and how elevating temperature alters the assimilation and utilisation of energy resources.

2.4.4 Summary

From the physiological measurements made for *Crassostrea gigas* it has been shown that filtration rate has the most significant effect on scope for growth (SFG). It is this process which provides energy for metabolism, but the quantity of the energy provided depends on a number of different factors. Food consists of particulate organic matter, detritus and bacteria. The quality and quantity of food is important in determining SFG as well as other factors such as current speeds and total particulate matter. There are also size effects, with a proportional rise in filtration rate as body size increase. All these factors were kept constant in this study and showed that temperature has the most significant effect on ingestion rates; salinity had much less effect. Absorption efficiency was more or less uniform across the environmental conditions tested, although at low temperature there was a slight increase as food stayed in the gut for longer. Respiration rates showed constant levels upto 15°C, with no significant differences between the temperatures; above 15°C there was a linear increase in respiration rates. This change represents a switch in physiology from winter to summer states, a phenomenon also described in other bivalves. Salinity changes provided maximum rates at 19‰ to 25‰. There was a small change in excretion rates at 15°C, above which they steadily increased. The scope for growth index gave optimum conditions at high temperature (20-25°C) and estuarine salinity (19-25‰) reflecting the ecological niche occupied by this species.

Body condition index (BCI) was inversely proportional to SFG and is probably more related to seasonal spawning cycles. At low BCI, usually after spawning, there are depleted energy resources so animals are frequently

susceptible to disease. The highest BCI was found in the range 10-15°C and 19-25‰. This study, as in previous studies, shows the need to use this index in conjunction with other indices, e.g. SFG, before firm conclusions about the health of the animal are drawn. Temperature was shown to have the most significant effect on energetic values. At low temperatures just enough is made available to maintain the animals' basal metabolism. 15°C provides the best conditions for the oyster with high levels of energy assimilated but little energy lost through respiration and excretion. At high temperature (25°C) a lot of energy is assimilated but there is also a higher energetic cost through respiration and excretion. Salinity has no significant effect on the energy partitioning of *C. gigas* but other external factors, e.g. particulate concentration, may also affect the animals.

CHAPTER THREE

COMPARISON OF *Bonamia ostreae* INFECTED

***Ostrea edulis*, NON-INFECTED *Ostrea edulis*,**

AND *Crassostrea gigas*

CHAPTER 3

COMPARISON OF *Bonamia ostreae* INFECTED *Ostrea edulis*, NON-INFECTED *O. edulis*, AND *Crassostrea gigas*

3.1 INTRODUCTION

In the previous chapter the physiology of *Crassostrea gigas* was described in detail with respect to changing temperature and salinity. This took as its model work described by Hutchinson and Hawkins (1992) on the variation in *Ostrea edulis* physiology in response to the natural range of temperature and salinity found in British waters. The extension of the approach to *C. gigas* is timely and appropriate, and is of significant interest to commercial enterprises since the Pacific oyster has superseded the native oyster as the bivalve of choice for farming. The need for this research into oyster physiology, immunology and metabolism has been prompted as natural and farmed populations were seriously affected by disease and resistant populations were sought. The survival of some populations of *O. edulis* when exposed to the parasite infection raises the possibility of some physiological or immunological parameter conferring a degree of immunity when compared with susceptible populations. By examining three populations of *Ostrea edulis*, before and after exposure to *Bonamia ostreae*, it was determined how their physiology influenced resistance to infection, and mortality rates. The data on *C. gigas* physiology was used for comparison as a possible means of establishing why this species is not susceptible to the parasite, as well as providing comparative data on the adaptability of bivalves to different conditions.

3.1.1 Bonamiasis in the U.K.

Although *Bonamia ostreae* infection was prevalent throughout Europe in the late 1970's its presence in the U.K. was not identified until 1982, in the rivers

Fal and Helford, and in the mid-Essex creeks (Hudson and Hill, 1991). Infection was found in Ireland in Cork Harbour in 1987 (McArdle *et al.*, 1991) although it is suspected that infection occurred earlier in the 1980s. No cure for the disease has been forthcoming and a full life cycle of the pathogen has only recently been described (Montes *et al.*, 1994). The strategies for preventing the spread of the disease mainly involved the control of animal and stock movements. The introduction of the Pacific oyster *Crassostrea gigas* into Europe (Grizel and Heral, 1990) and Britain (Utting and Spencer, 1992) has enabled the oyster fishing industry to sustain itself, but there is still a demand for the native flat oyster. Natural populations of *Ostrea edulis*, such as the Solent population, have been carefully monitored for *B. ostreae* infection, and so far the disease has been at low level, whilst populations in Wales and Scotland have been maintained as disease free.

Anecdotal evidence has suggested that stress is a pre-disposing factor to disease infection, so much research has been aimed at quantifying physiological, biochemical and/or immunological parameters of the oyster. All these methods were employed in a comprehensive study of the baseline responses of *O. edulis* to temperature and salinity stress (Hawkins and Hutchinson, 1990) and a full set of physiological measurements for *C. gigas* are presented in chapter 2. However, by identifying optimal and detrimental conditions better management of the species can be achieved and disastrous losses can be avoided. Work has already begun on trying to develop strains of *O. edulis* resistant to bonamiasis. Survivors of the 1987 infection in Ireland have produced F1 and F2 generations - the F1 generation shows no selective advantage for *B. ostreae* resistance and the F2 are still being tested (Hugh-Jones, *pers. comm.*). A trial to build up resistant strains has been established in the Netherlands (van Banning, 1991). In the U.K., the MAFF Fish Diseases Laboratory, Weymouth established a field trial investigating the responses of three British populations of *O. edulis* to a known *B. ostreae* infected area - the river Helford. Animals from the Solent population, on the south coast of England, from Conwy, north Wales, and from Loch Sween, Scotland (figure 3.01), were used in this investigation. For meaningful, direct comparisons of the populations, physiological measurements were made for each under the same environmental



Figure 3.01 Sample sites around the U.K.

conditions. These were made before deployment and after a period of time in the infected area. Details of genetic differences are described in chapter 5.

3.1.2 Origins of the Conwy *O. edulis* population

The *O. edulis* used in these experiments were reared in the hatchery at the Ministry of Agriculture, Fisheries and Food (MAFF) laboratory, Conwy. The animals were taken from the Menai Strait bonamia-free stock (Spencer, *pers. comm.*). The earlier parentage of this stock is unlikely to be local; in the early 1980s broodstock was supplied from a number of areas including the Solent, Poole Harbour, Essex, and the Fal. This practice has now been discontinued.

3.1.3 Origins of the Loch Sween *O. edulis* population

The origins of this stock are also quite varied. A 16th century map of the area marked on the Linne Mhuirich arm of the loch '*This creek is good for Oysters*'. By the 17th century it was common for oysters to be collected in the more northerly lochs and relaid in Loch Sween and another loch. Various relayings from as far afield as France were carried out in the Argyll area (Berry, *pers. comm.*).

In this century oyster seed was bought from the Norwegian pols at Austevoll and laid in Linne Mhuirich during the 1960s, and Norwegian seed was ongrown at a nearby mussel farm in the late 1970s. It is not known whether these stocks mixed with the stocks already present. Between 1985 and 1987 seed from larvae obtained from Guernsey Seafarms have been ongrown at Loch Sween. Adult native oysters have been taken from natural beds around the island of Ulva, the west of Mull, Loch Creran, and in 1990 from the last remaining Shetland oysters, and have all been induced to spawn in Loch Sween.

3.2 MATERIALS AND METHODS

3.2.1 Collection and Maintenance of Oysters

Ostrea edulis were obtained from Loch Sween, Scotland, Conwy, north Wales, and from the Solent on the south coast of England (figure 3.01). These animals were to be used for the investigation of resistance to *Bonamia ostreae*, and were placed in a former oyster rearing site at Calamansack Creek in the river Helford, Cornwall an area known to have *Bonamia ostreae*. Three hundred oysters of approximately the same shell weight from each population were divided in to groups of fifty animals and placed in Netlon™ cages with identification tags. The cages of oysters were placed at random on trestles at the low water spring tide mark; the maximum water temperature range at the site over the experimental period was 5.7 - 15.8°C and the salinity range was 22 - 33‰ (data supplied by NRA). The experiment began on 6 November 1990, and the animals were sampled by the FDL, Weymouth at low water spring tides after 6 and 18 months.

These animals were either transferred back to the Department of Oceanography, University of Southampton to an enclosed tidal system under conditions similar to those in the field, or they were sacrificed for histological examination for *Bonamia* infection at FDL, Weymouth. After 7-10 days acclimation to the aquarium system, at a temperature of 15°C and a salinity of 33‰ (which were close to the field conditions), the former group was analysed for its physiological characteristics. Acclimation under these conditions was chosen to eliminate seasonal differences, without unduly stressing the animals from their field conditions. The enclosed system used for the animals quarantine had a 200 l capacity, with sea water re-circulated from a reservoir tank to a header tank, before returning through the sample tank to the reservoir. A water bath was used to pre-cool or pre-heat the water to the appropriate temperature, and the whole system was maintained in a 15°C constant temperature room.

During the course of the analyses, there was no significant, observable build up of waste products in the system. Animals were fed with the same algal regime as described in section 2.2.1 and the scope for growth components were measured using the methods described in 2.2.2. Animals sampled after six months have been labelled 1991, and those sampled after fourteen months are labelled

1992. Results from histological examination by the FDL, Weymouth indicated that not all of the oysters sampled were infected, but all effluent from the tanks as well as the animals shells were disinfected with sodium hypochlorite (minimum 8% free chlorine).

3.2.2 Haemocytic Effects of Elevated Lactate Concentration

Ostrea edulis from the Solent, which were tidally exposed, have been shown to accumulate the toxic metabolite lactic acid (Hawkins and Hutchinson, 1990). Investigations were made to analyse the manner in which lactate affects the cellular immunological responses, with a view to possibly extending this avenue of research.

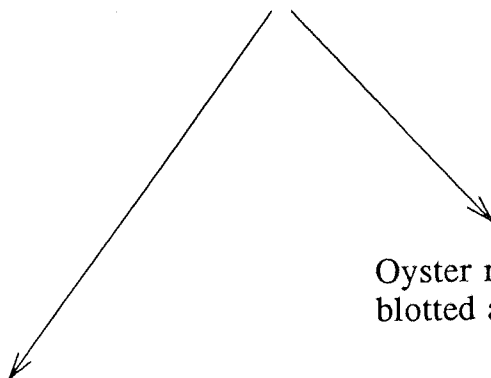
A 1 μM solution of lithium lactate in filtered sea water (filtered through a 0.1 μm membrane filter) was made by serial dilution. Lithium lactate was used because the sodium salt would increase the sodium concentration of the sea water, and the calcium salt has a known inhibitory effect on microtubule formation, and so would inhibit cell movement. Previous experiments have shown that chronically exposed (on a tidal cycle) oysters produce approximately 0.09 $\mu\text{mol g}^{-1}$ wet tissue weight of lactate (Hawkins and Hutchinson, 1990). This was used as the concentration for amoebocyte challenge.

Oysters from the Solent *O. edulis* population were sampled from ambient aquarium conditions (13-19°C/31-33‰) at random and a haemolymph sample (approximately 300 μl) was taken using a drawn-out Pasteur pipette. The oyster was then removed from its shell, blotted to remove excess water, and weighed. This weight was used to calculate the amount of 1 μM lactate sea water required for dilution to give an equivalent concentration of 0.09 $\mu\text{mol g}^{-1}$, so that 50 μl of whole blood sample was mixed with 50 μl of diluted lactate sea water. For control blood amoebocyte measurement, 50 μl of whole blood was mixed with an equal amount of normal, filtered sea water (i.e. no lactate was added). The pH of mantle fluid samples taken for experimental and control animals was measured using a pH meter, as was the pH of the lactate sea water medium. Samples were put on a haemocytometer and a suitable aggregation of amoebocytes was identified at x20

1 μM solution of lithium lactate in sea water prepared by serial dilution

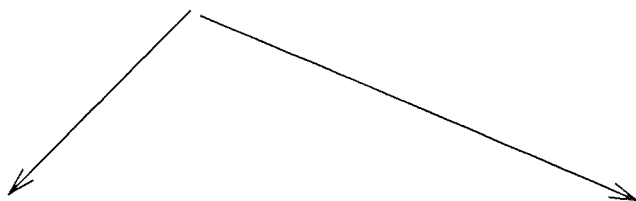


Sample of whole blood taken from oyster



Oyster removed from shell,
blotted and weighed

Diluted with normal sea water to give concentration
of $0.09 \mu\text{mol g}^{-1}$ wet weight



Experimental

50 μl sample mixed with
50 μl lactate sea water



Sample thoroughly mixed,
placed on haemocytometer,
observed and recorded

Control

50 μl mixed with
50 μl normal sea water



Sample thoroughly mixed,
placed on haemocytometer,
observed and recorded

Figure 3.02 Protocol for lactate challenge of amoebocytes.

magnification, and then observed and recorded at x40. This protocol is summarised in figure 3.02.

3.2.3 Statistical Analysis of Results

The results of laboratory experiments were expressed as mean values \pm standard error, unless otherwise stated. The number of observations at each datum point for the scope for growth measurements was 12; the effects of elevated lactate were measured for $n=6$ at each datum point. Where appropriate data were analysed using an unpaired t-test (or a Mann-Whitney test as a non-parametric alternative) for comparing two conditions, or a standard ANOVA (or a Kruskal-Wallis test as a non-parametric alternative) for comparing more than two conditions. Pearson product-moment correlations were performed to measure the association between variables; this test assumed the data were normally distributed and did not assign dependent and independent variables. Data for the dry weight versus physiological rate were transformed using a standard natural-log transform before regressions were calculated.

3.3 RESULTS

3.3.1 Scope for Growth in *O. edulis* populations

Animals were fed with a particulate concentration of approximately 25,000 cells per cm³; the filtration rates measured for this concentration are indicated in figure 3.03. The Conwy animals used were the smallest of the three populations, with no significant difference between the 1991 (mean tissue dry weight = 0.32 g) and 1992 (mean tissue dry weight = 0.23 g) animals ($t = 0.4814$, $P < 0.05$). The 1992 Loch Sween oysters (mean tissue dry weight = 0.79 g) showed a higher rate of filtration than the animals sampled in 1991 (mean tissue dry weight = 0.92 g) but this difference was not significant ($P > 0.05$). Although they had a mean dry weight similar to the Solent stock (mean tissue dry weight = 0.79 g), the filtration rate was much larger than that demonstrated by the English animals. The amount of variation in filtration rate observed meant that differences between populations were not significant ($P > 0.05$). From the correlation results, there was an apparent exponential size effect for filtration rates when Conwy and Loch Sween oysters were combined ($r = 0.46$; $P = 0.004$) - the dependent variable filtration rate tended to decrease as the independent variable size increased. When considered separately there was a linear relationship for each group of animals.

The absorption efficiencies of the three populations are represented in figure 3.04; there was no significant correlation between the size of the animals and their efficiency of absorption ($r = 0.25$). Although the Solent animals had the lowest ingestion rate (figure 3.03) they had a higher (but not statistically significant) absorption efficiency than 'healthy' 1991 Loch Sween oysters. For the purposes of this discussion, the term 'healthy' is used to denote animals sampled in 1991, which were shown by the FDL, Weymouth to have no parasitic infection. Some of the animals sampled in 1992 were found to have *B. ostreae* infection, so animals taken in this year have been termed 'diseased'. The increase in filtration rate in Loch Sween animals, between 1991 and 1992 stocks, was matched by an increasing absorption efficiency. The change in this efficiency observed for the Conwy population was not statistically significant ($P > 0.05$), with both samples absorbing close to the maximum of labile material available to them. There was no significant relationship between body size and absorption efficiency (figure 3.04b;

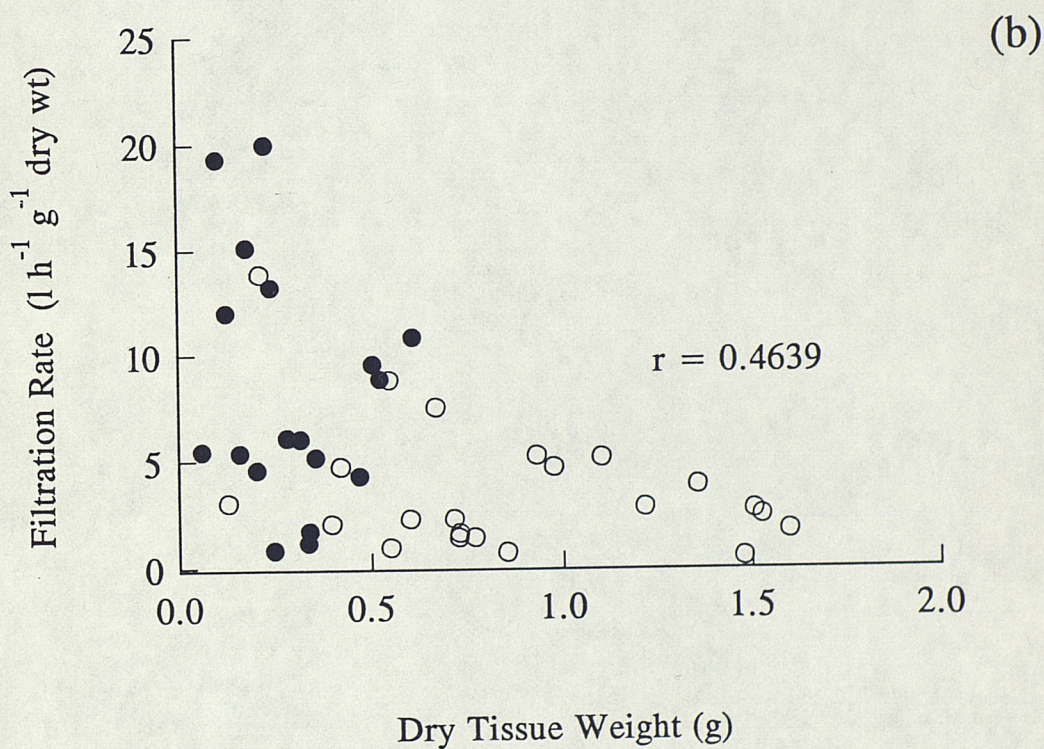
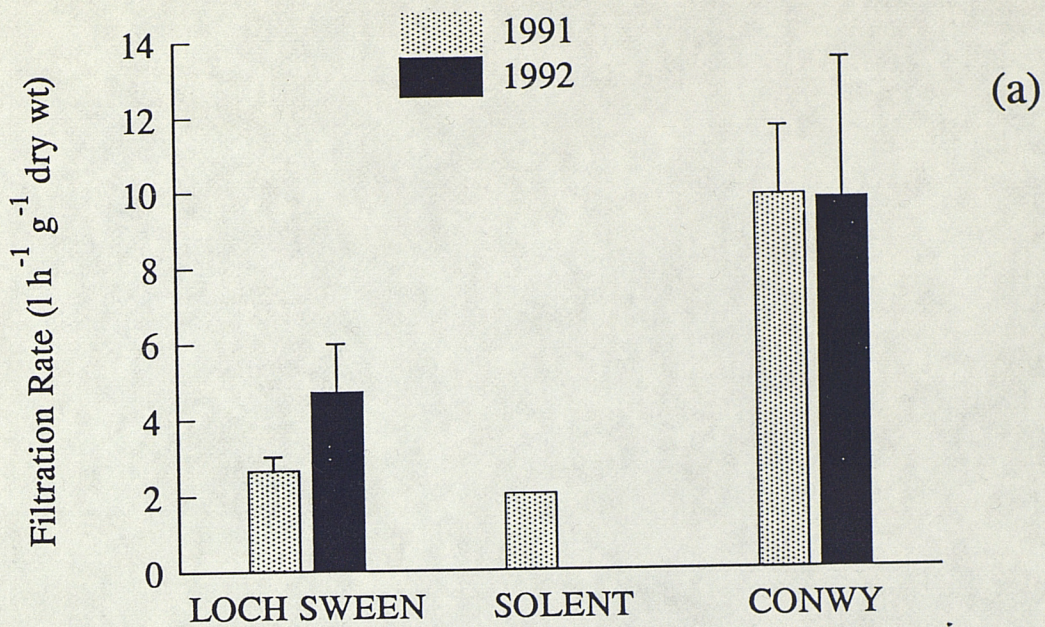


Figure 3.03 (a) Filtration rate ($\text{l h}^{-1} \text{g}^{-1}$ dry weight) in three populations of *Ostrea edulis*. Mean \pm SE, $n = 12$ at each point. (b) Filtration rate versus dry weight correlation for Loch Sween (\circ) and Conwy (\bullet) oysters.

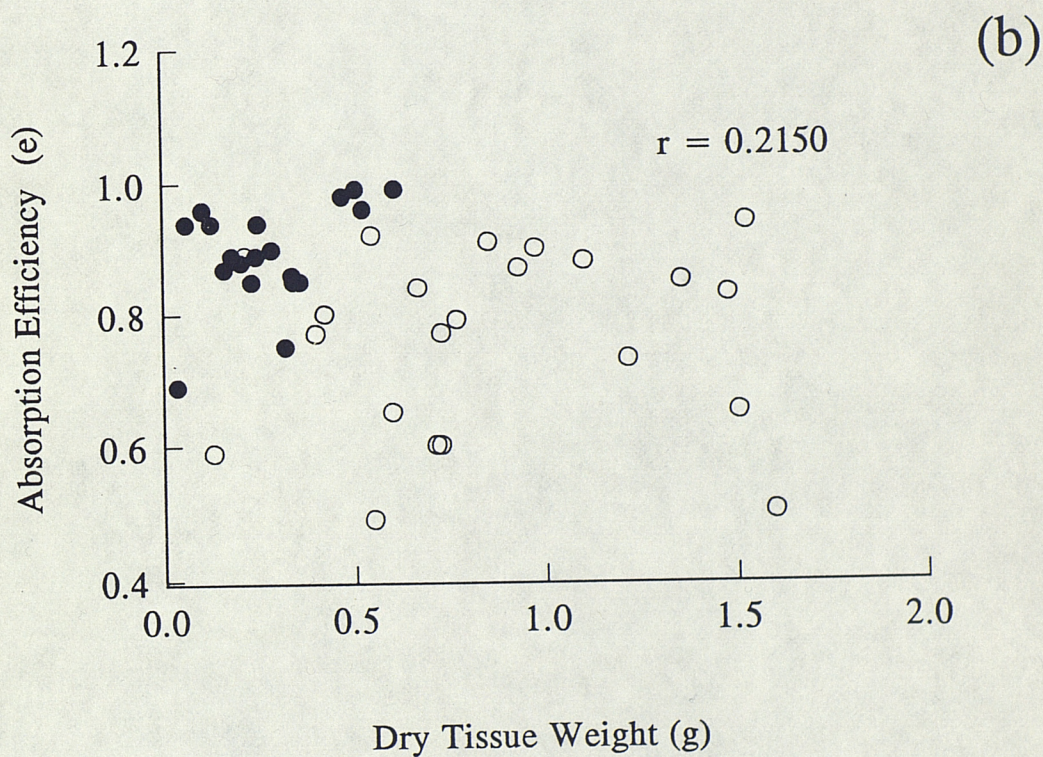
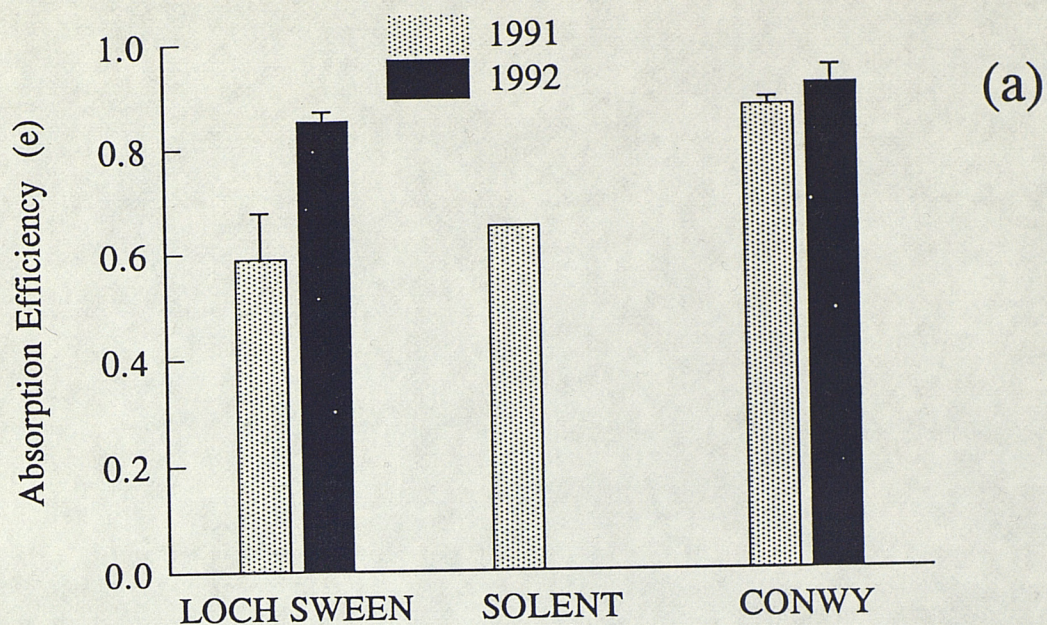


Figure 3.04 (a) Absorption efficiency (e) for three populations of *Ostrea edulis*. Mean \pm SE, $n = 12$ at each point. (b) Absorption efficiency versus dry weight correlation for Loch Sween (\circ) and Conwy (\bullet) oysters.

$P > 0.05$).

The results for absorbed energy consumed by respiration are shown in figure 3.05. There was variability in the 'healthy' (1991) populations of *Ostrea edulis* which may have been a reflection of inherent differences. One-way ANOVA showed that there were no statistically significant differences between any of the observed results ($P > 0.05$). However, for the 1992 samples, the respiration rates decreased in Loch Sween oysters and increase in the Conwy animals. The individual animals gave some variable results as reflected by the large standard error (the level of infection could cause this variability). As with filtration rate there was a significant inverse, exponential relationship between dry weight and respiration rate ($r = 0.49$; $P < 0.001$).

Nitrogenous excretion (figure 3.06) proved to be much higher in the Conwy animals than in other populations. The healthy animals from 1991 had significantly higher excretion rates than either of the Scottish groups ($P < 0.05$) but no other comparisons proved to be statistically significantly different ($P > 0.05$). These animals were the smallest used, with the larger animals having a correspondingly lower rate of excretion. The correlation coefficient for dry tissue weight - excretion rate was higher than for other physiological measurements ($r = 0.57$) and again showed a significant inverse, exponential relationship ($P < 0.001$).

The Scope for Growth (SFG) index is shown in figure 3.07, with a similar plot to that given by filtration rate (figure 3.03). The diseased Loch Sween animals exhibited an apparent increase in SFG over their healthy counterparts. The Conwy population had a drop in SFG which could be attributed to increased respiration with no additional energy intake. There were significant differences between both Conwy groups and the healthy Scottish oysters ($P < 0.05$) but not between any other comparisons ($P > 0.05$). The error bars for the Conwy groups were still large (healthy = 189.67 ± 38.51 ; diseased = 170.93 ± 52.19) resulting in the lack of significant SFG values ($P > 0.05$). There was a gradual rise in the SFG values between the Loch Sween, Solent and Conwy populations as the mean tissue dry weight decreased. When this was plotted against the SFG values on individual animals (for Loch Sween and Conwy only) as with the components of this index

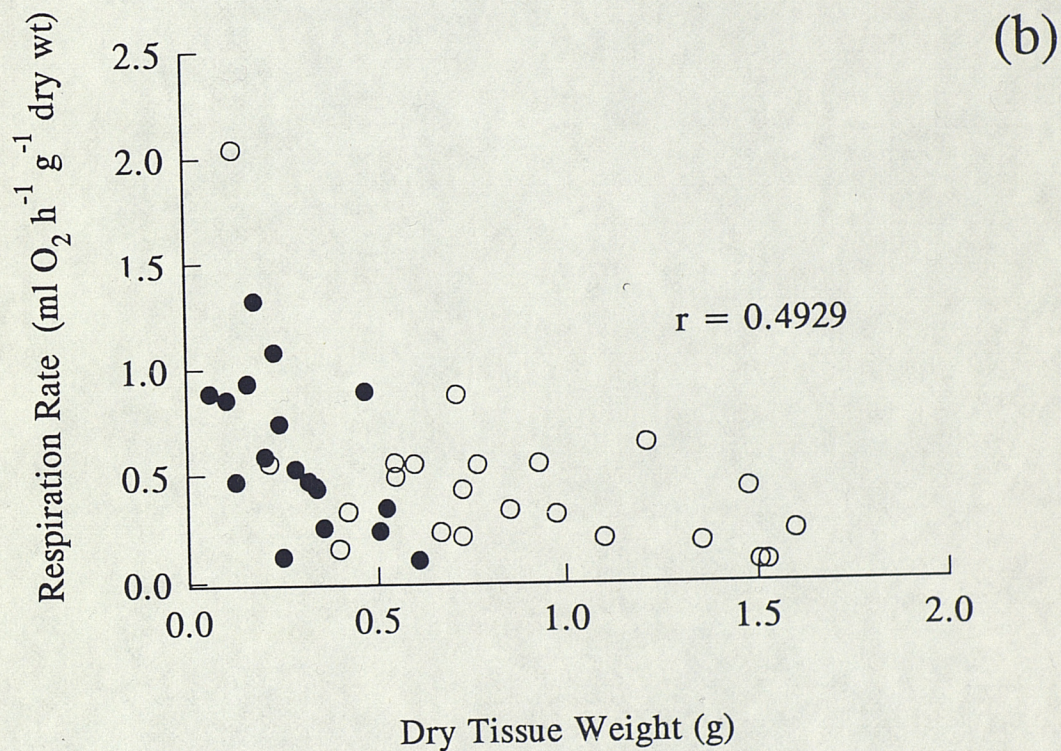
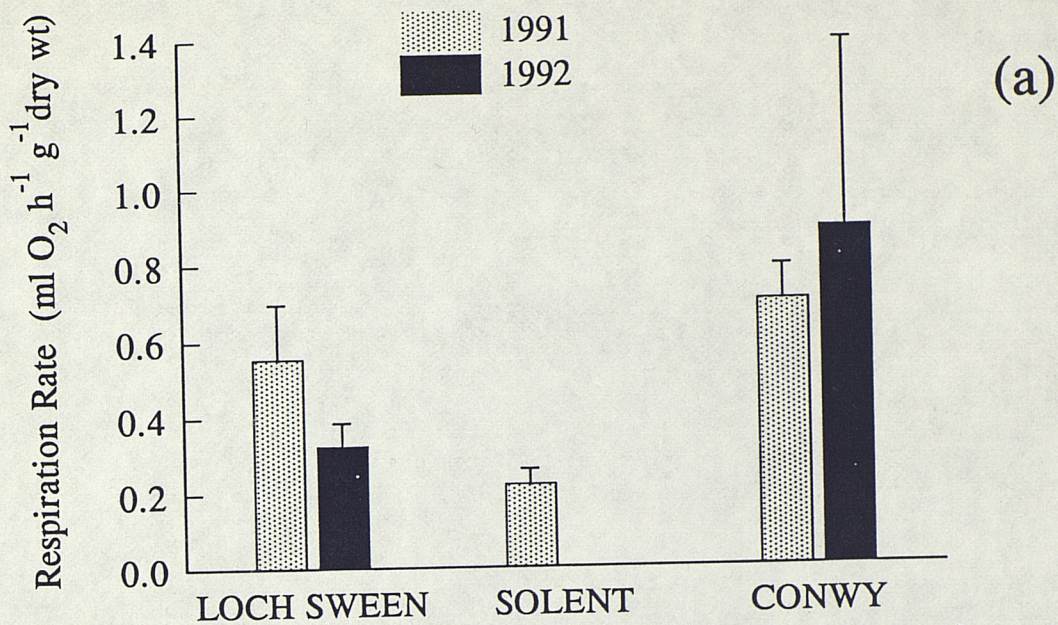


Figure 3.05 (a) Respiration rate (ml O₂ h⁻¹ g⁻¹ dry weight) for three populations of *Ostrea edulis*. Mean \pm SE, n = 12 at each point. (b) Respiration rate versus dry weight correlation for Loch Sween (○) and Conwy (●) oysters.

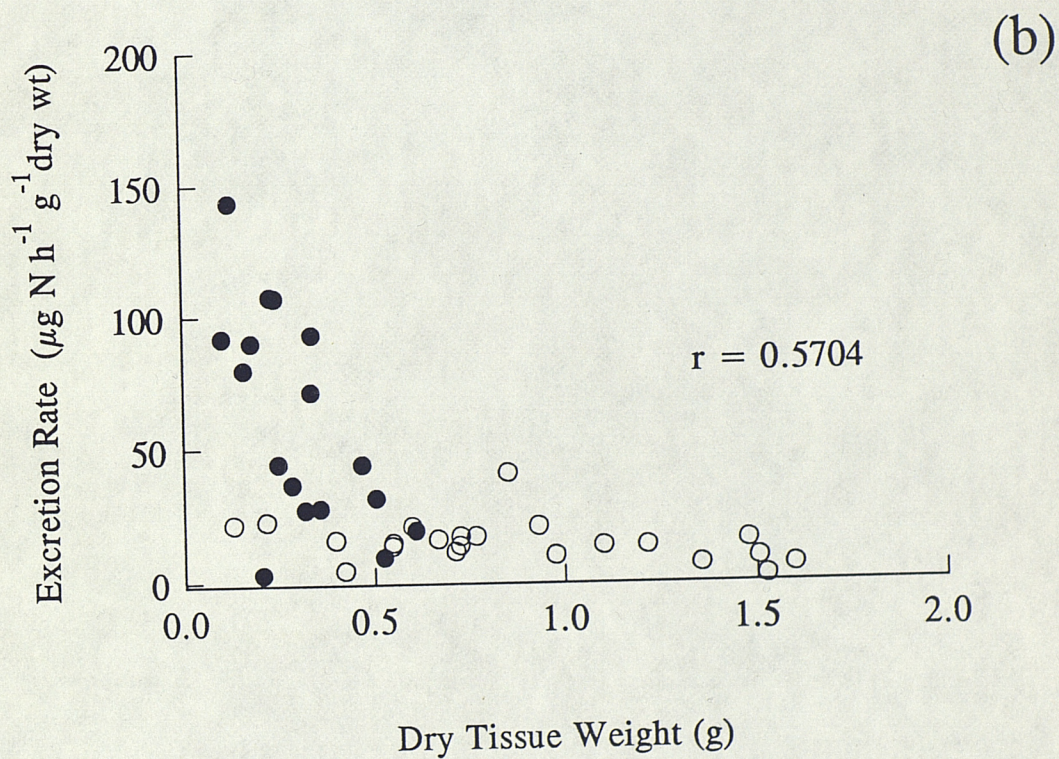
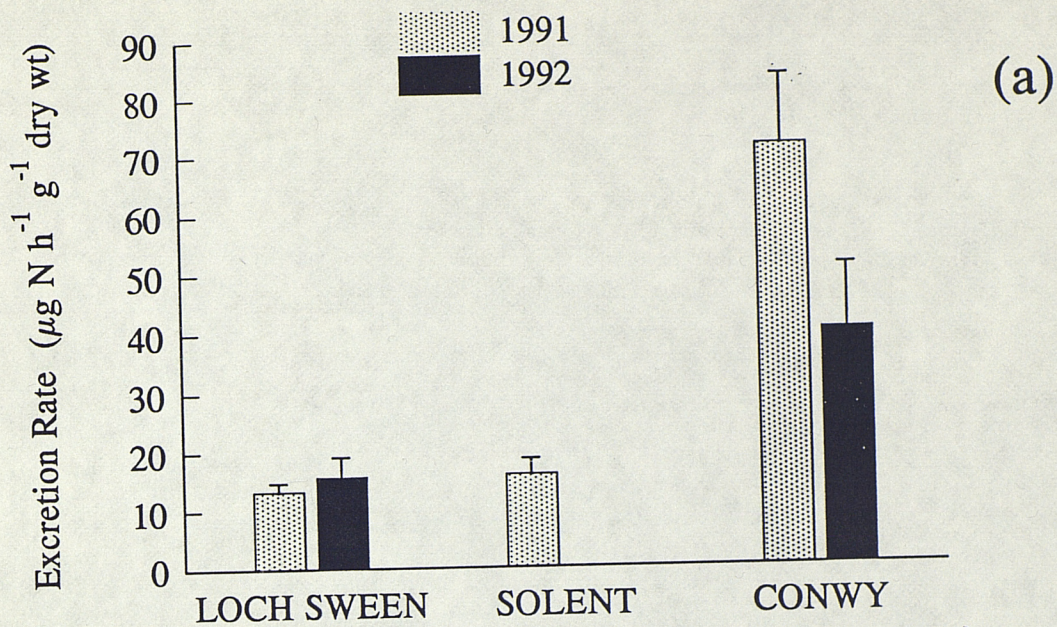


Figure 3.06 Excretion rate ($\mu\text{g N h}^{-1} \text{g}^{-1}$ dry weight) for three populations of *Ostrea edulis*. Mean \pm SE, $n = 12$ at each point. (b) Excretion rate versus dry weight correlation for Loch Sween (○) and Conwy (●) oysters.

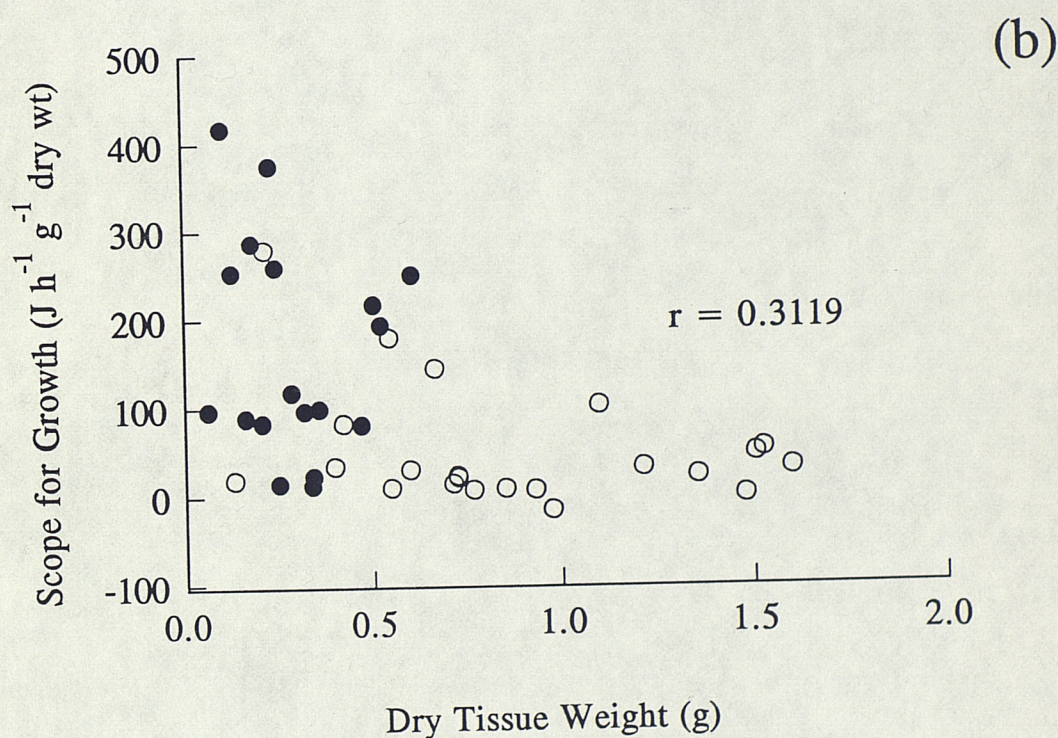
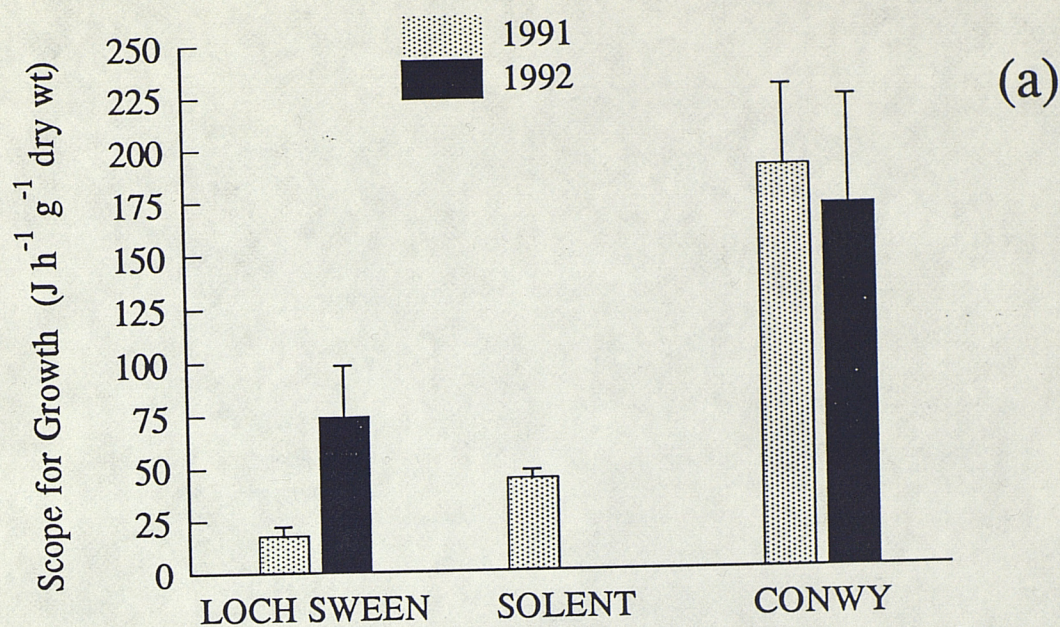


Figure 3.07 (a) Scope for Growth ($\text{J h}^{-1} \text{g}^{-1}$ dry weight) for three populations of *Ostrea edulis*. Mean \pm SE, $n = 12$ at each point. (b) Scope for Growth versus dry weight correlation for Loch Sween (\circ) and Conwy (\bullet) oysters.

there was a significant exponential relationship with SFG decreasing with increasing body size ($r = 0.392$; $P = 0.015$).

3.3.2 Body Condition Index in *O. edulis* Populations

In figure 3.08 the body condition index (BCI) for each population is represented. If these figures are compared with those of SFG (figure 3.07) it is seen that there is an apparent inverse relationship and this is supported by a Pearson product-moment correlation ($r = -0.352$; $P = 0.024$). There was a statistically significant difference between the two 1992 groups ($P < 0.05$) but not between any other comparisons. This index was calculated using the dry tissue weight and consequently showed a good, positive correlation-regression plot (figure 3.08b; $r = 0.731$, $P < 0.001$).

3.3.3 Energy Partitioning in *O. edulis* Populations

The raw data for Solent oysters was unavailable and so were not included in statistical tests for the fates of ingested energy, shown for the three populations of *Ostrea edulis* at 15°C/33‰ in figures 3.09 to 3.11. These proportions were calculated only for the healthy 1991 results. From the figures it can be seen that there is a large proportion of unused energy with the Conwy animals losing much less of their ingested resources than the other animals. The Scottish oysters lost significantly more energy as faeces and pseudofaeces than the Conwy population or the *Tapes* animals, to be discussed in section 4.4.2 ($P < 0.05$), but individual variability meant that comparisons with *C. gigas* oysters (figure 2.09) were not significant ($P > 0.05$).

The assimilated energy, before it is available for somatic or reproductive growth, is used in respiration and ammonia excretion. Only a very small amount of this energy is lost through the latter process although significant differences were seen between the Conwy population (figure 3.10) and *C. gigas* & *T. philippinarum* ($P < 0.05$), and between these two species and the Loch Sween oysters (figure 3.11; $P < 0.05$). Although no raw data are available for the Solent

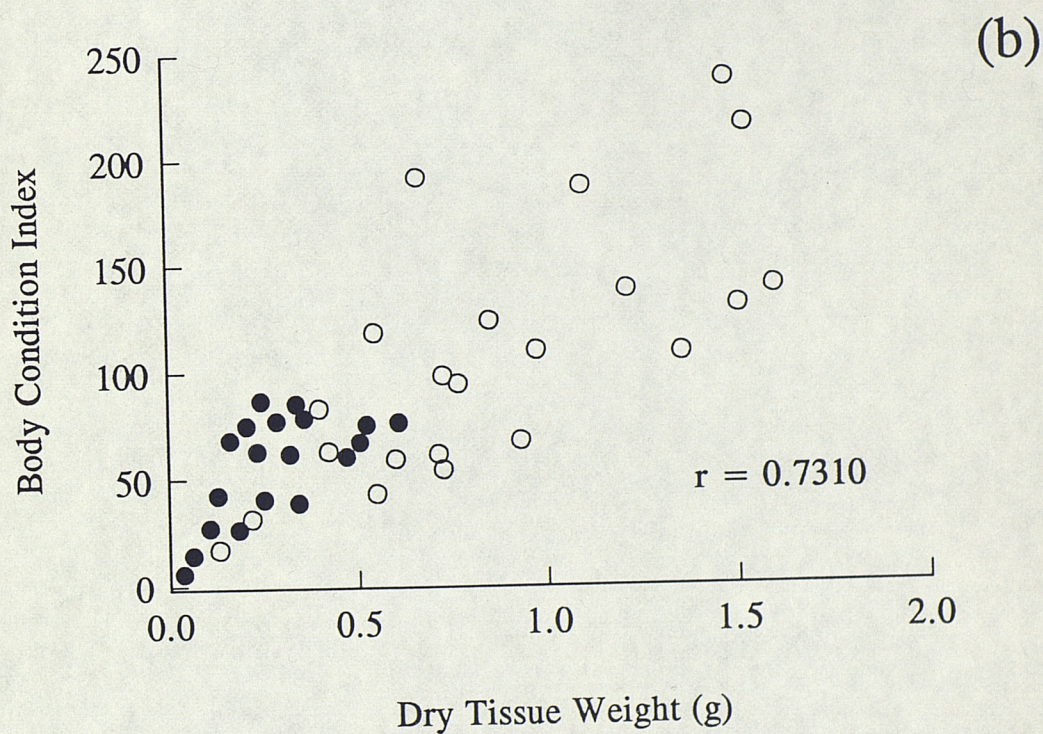
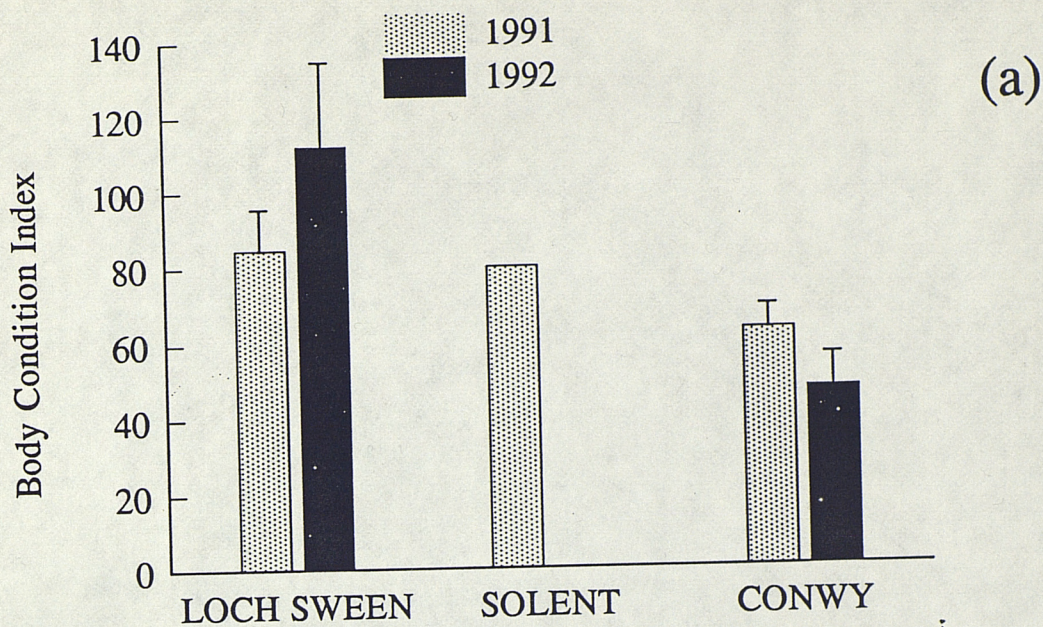


Figure 3.08 (a) Body Condition Index for three populations of *Ostrea edulis*. Mean \pm SE, $n = 12$ at each point. (b) Body Condition Index versus dry weight correlation for Loch Sween (\circ) and Conwy (\bullet) oysters.

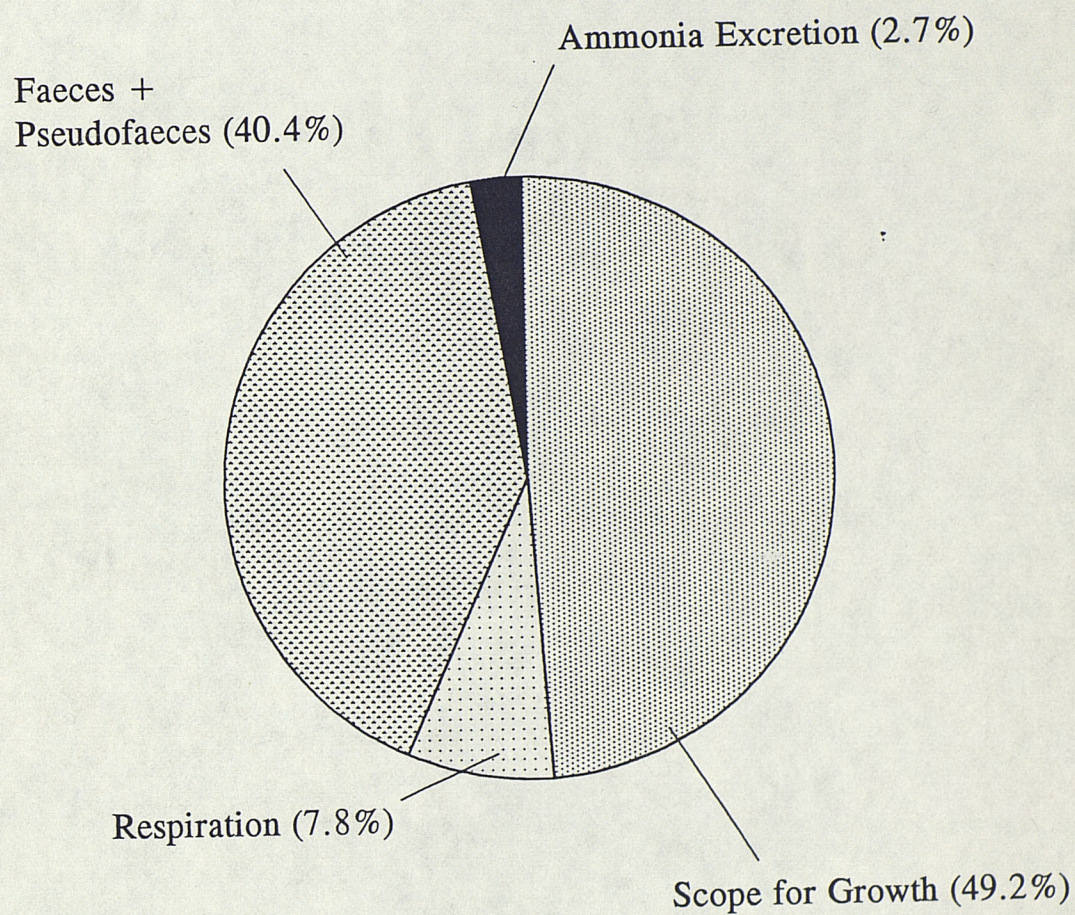


Figure 3.09 Partitioning of ingested energy in Solent *Ostrea edulis* at 15°C and 33‰. Mean values, n = 12.

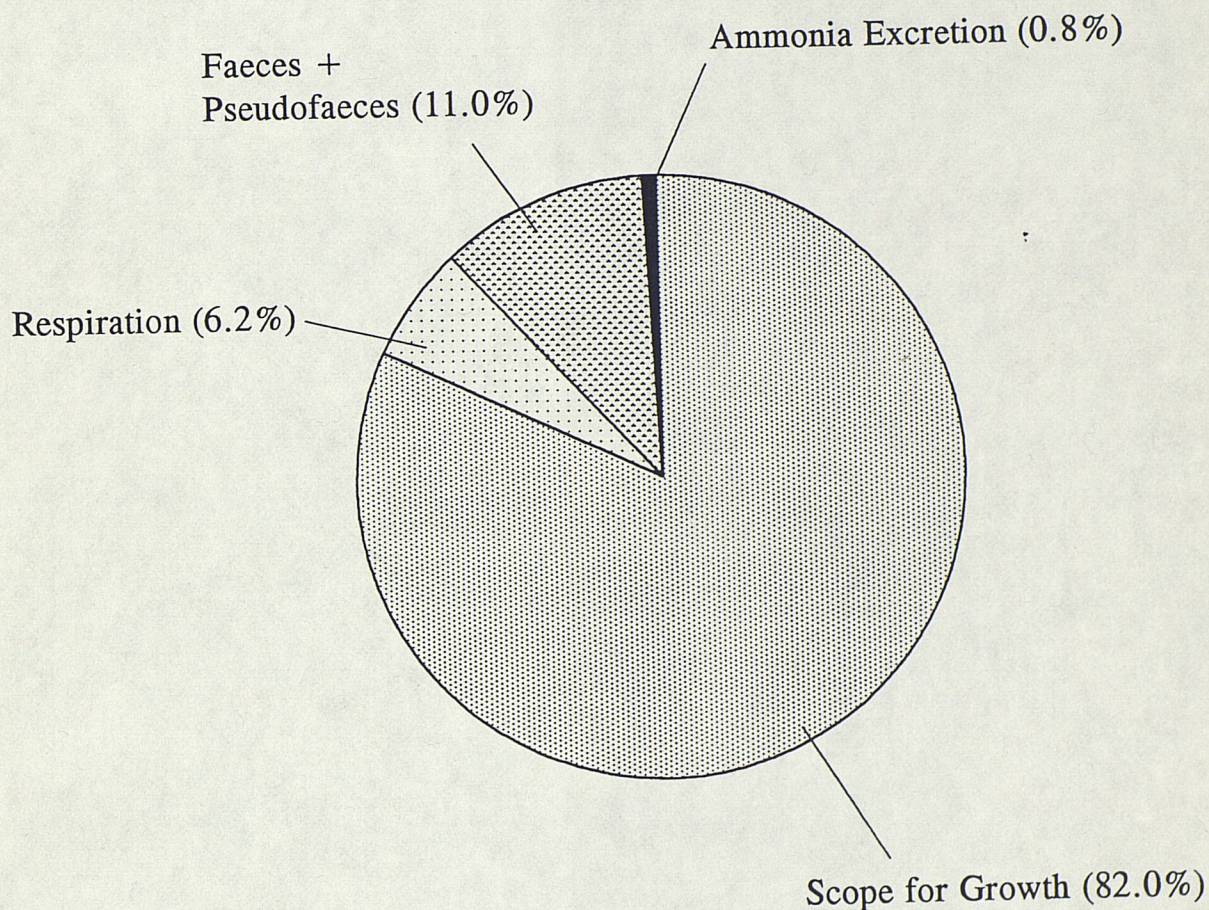


Figure 3.10 Partitioning of ingested energy in Conwy *Ostrea edulis* at 15°C and 33‰. Mean values, n = 12.

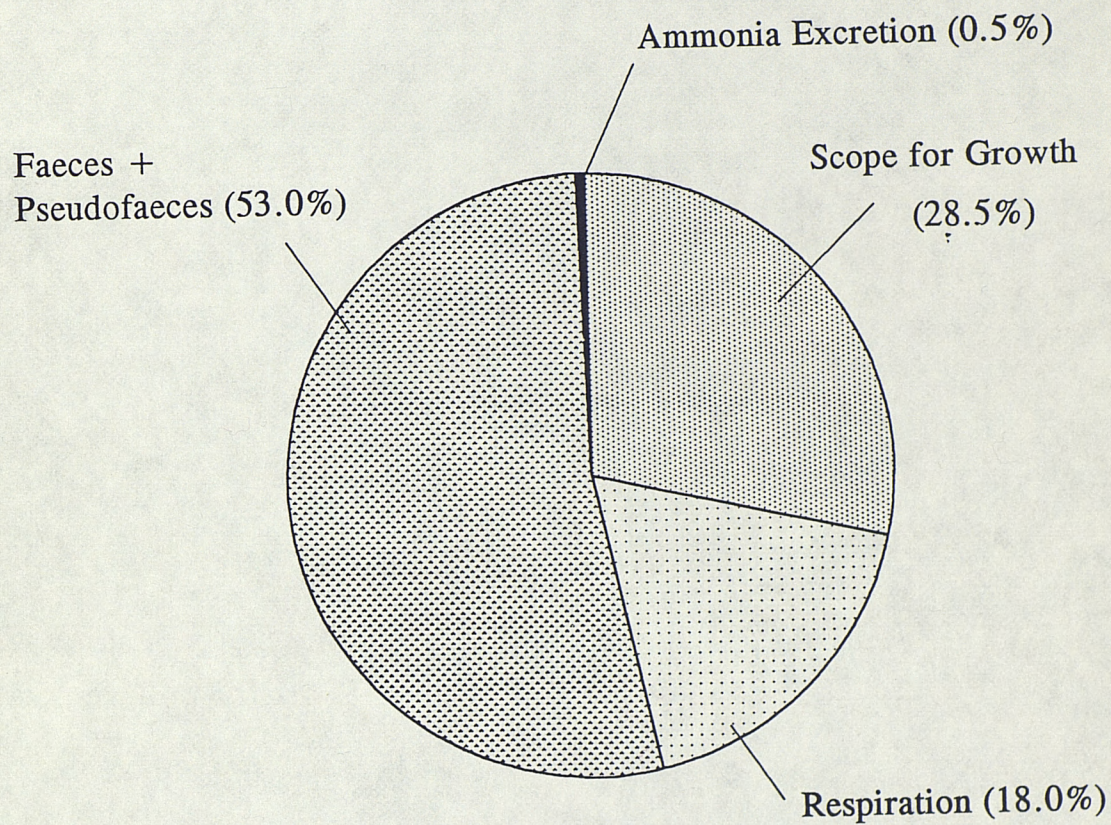


Figure 3.11 Partitioning of ingested energy in Loch Sween *Ostrea edulis* at 15°C and 33‰. Mean values, n = 12.

population (figure 3.09) this demonstrated the largest proportion of energy spent in ammonia excretion (2.7% of the total ingested energy). The respiration differences were much greater. *C. gigas* lost half the amount of energy that the Conwy and Solent populations did, and although it had a similar proportion to that of *T. philippinarum* it still showed a significant difference ($P < 0.05$). Loch Sween oysters had more than twice the energy lost in respiration (18% of the total ingested energy) than the other groups studied and consequently showed significant differences from all the groups ($P < 0.05$).

The remaining assimilated energy is available for scope for growth. Again there was an inverse size relationship with the smaller animals showing much higher SFG than the larger animals. This was also demonstrated in the absolute energetic values presented in section 3.3.1. The Scottish oysters had significantly less energy for SFG than the other groups ($P < 0.05$) but comparisons between these other populations showed statistically non-significant differences ($P > 0.05$).

3.3.4 Comparison of Solent *O. edulis* and *C. gigas* Physiology

In figure 3.12 the filtration rates of *O. edulis* (3.12b) and *C. gigas* (3.12a) are compared directly. The two patterns are highly significantly different ($P < 0.001$), with *C. gigas* having filtration rates in the range 0.71 to 21.28 l h⁻¹ g⁻¹ dry weight compared with the *O. edulis* range of 0.10 to 5.20 l h⁻¹ g⁻¹ dry weight. The optimum conditions for filtration rate in *C. gigas* occurred at high temperature (25°C) but at reduced salinity (22-25‰), whereas the optima for *O. edulis* were 25°C and 33‰. There was also a significant difference ($P < 0.001$) between the absorption efficiencies of the two species (figure 3.13). *C. gigas* exhibited a maximum range of absorption efficiency between 0.56 and 0.91 which was slightly better than the range of 0.49 to 0.80 for *O. edulis*. Optimum efficiency for *C. gigas* occurred at lower temperatures (5-10°C) and at a similar salinity to the optimum for filtration rate (25‰).

Comparison of the respiration rates (figure 3.14) proved to be non-significant ($P = 0.126$), although there was a marked difference in the distribution of maximum rates. *C. gigas* had a maximum range of 0.11 to 2.79 ml O₂ h⁻¹ g⁻¹

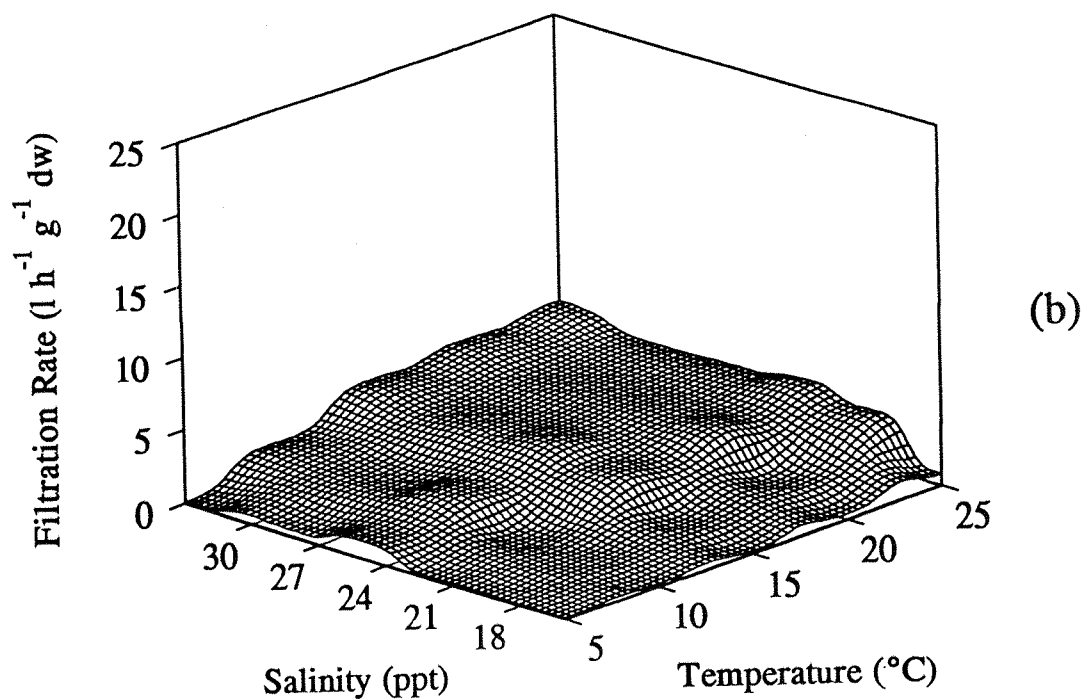
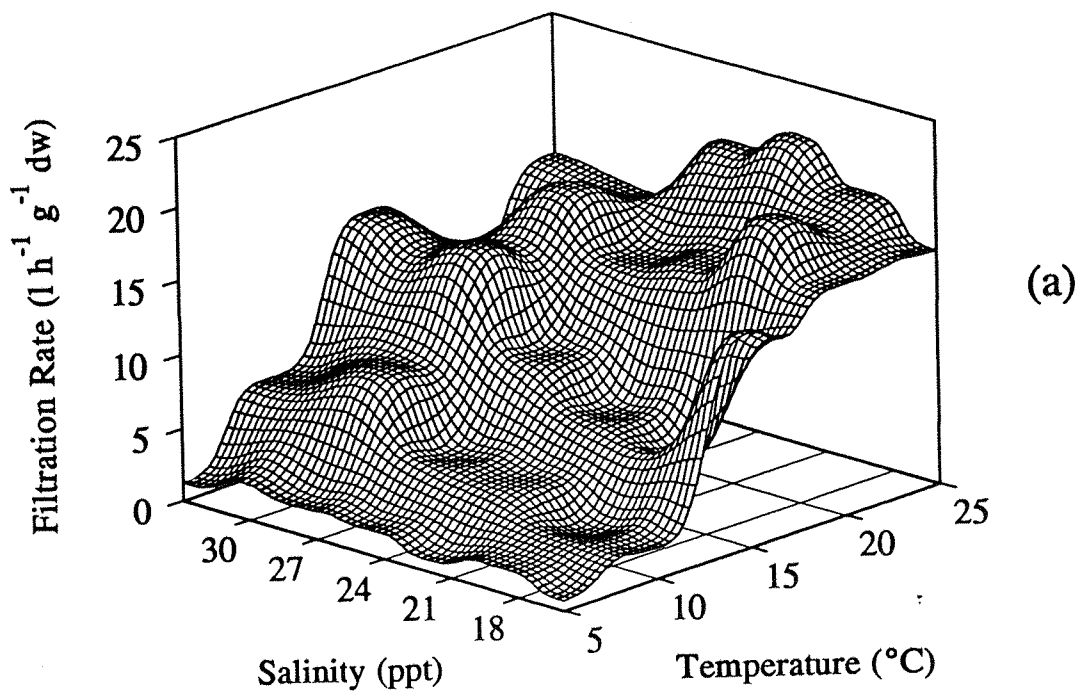


Figure 3.12 Comparison of the effects of temperature and salinity on the filtration rates ($\text{l h}^{-1} \text{g}^{-1}$ dry weight) of (a) *Crassostrea gigas* and (b) *Ostrea edulis*. Mean values, $n = 12$ at each point.

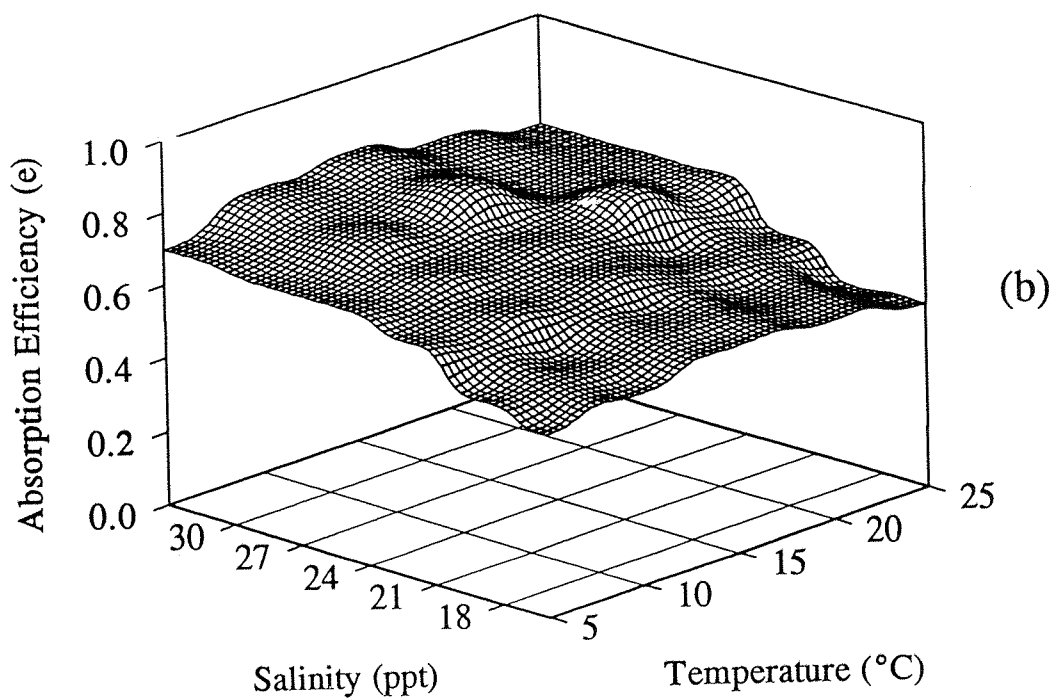
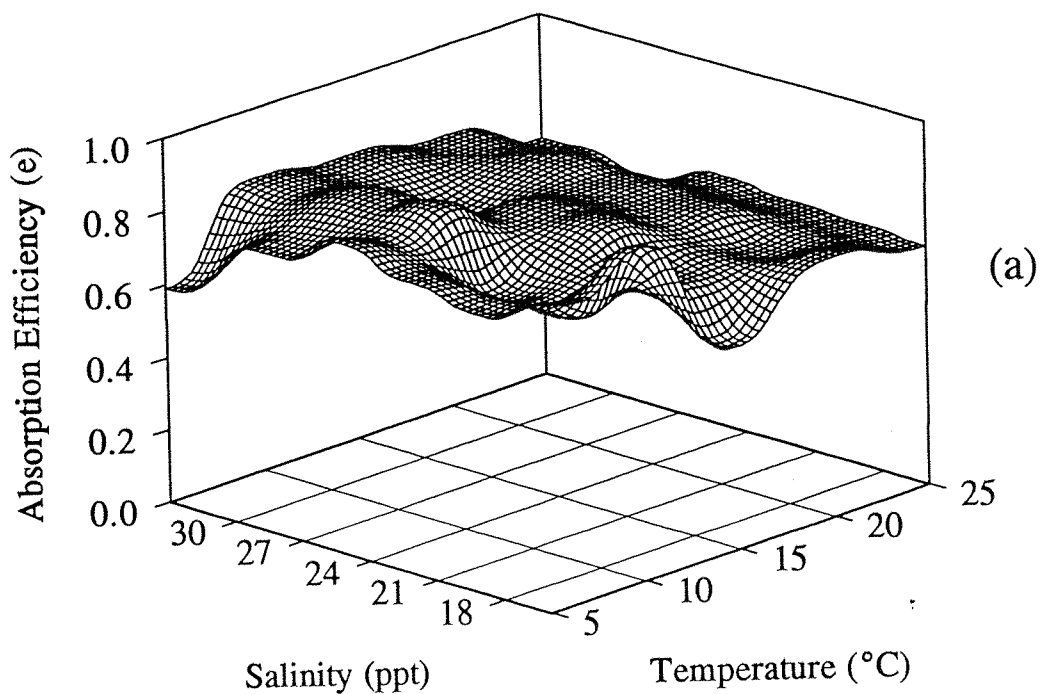


Figure 3.13 Comparison of the effects of temperature and salinity on the absorption efficiency (e) of (a) *Crassostrea gigas* and (b) *Ostrea edulis*. Mean values, $n = 12$ at each point.

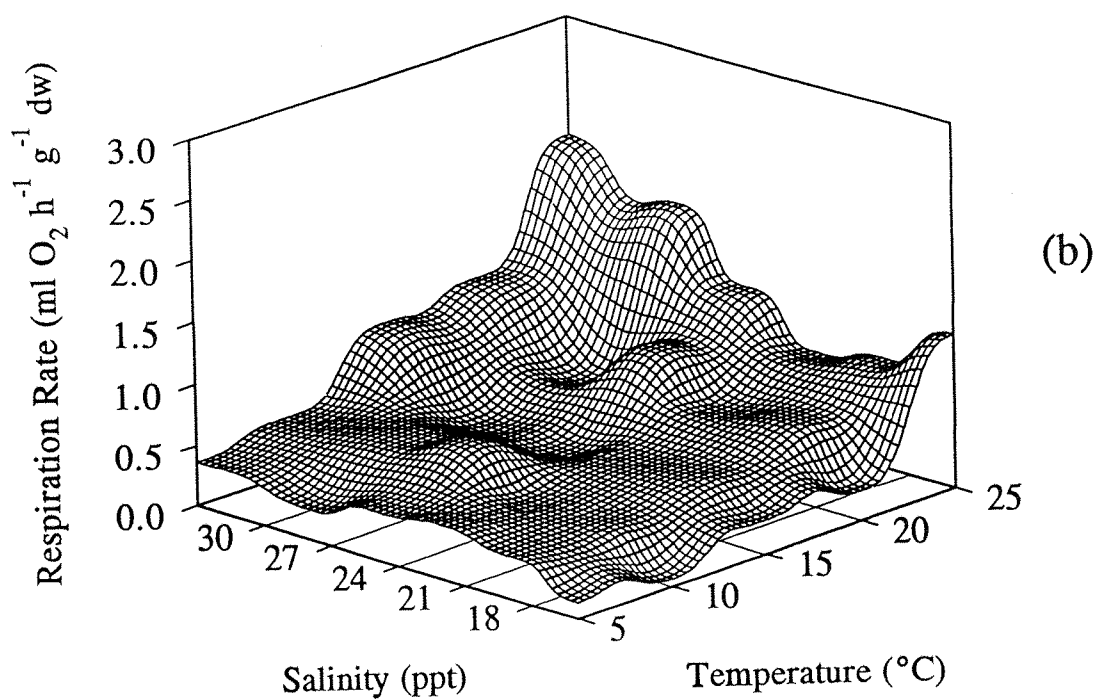
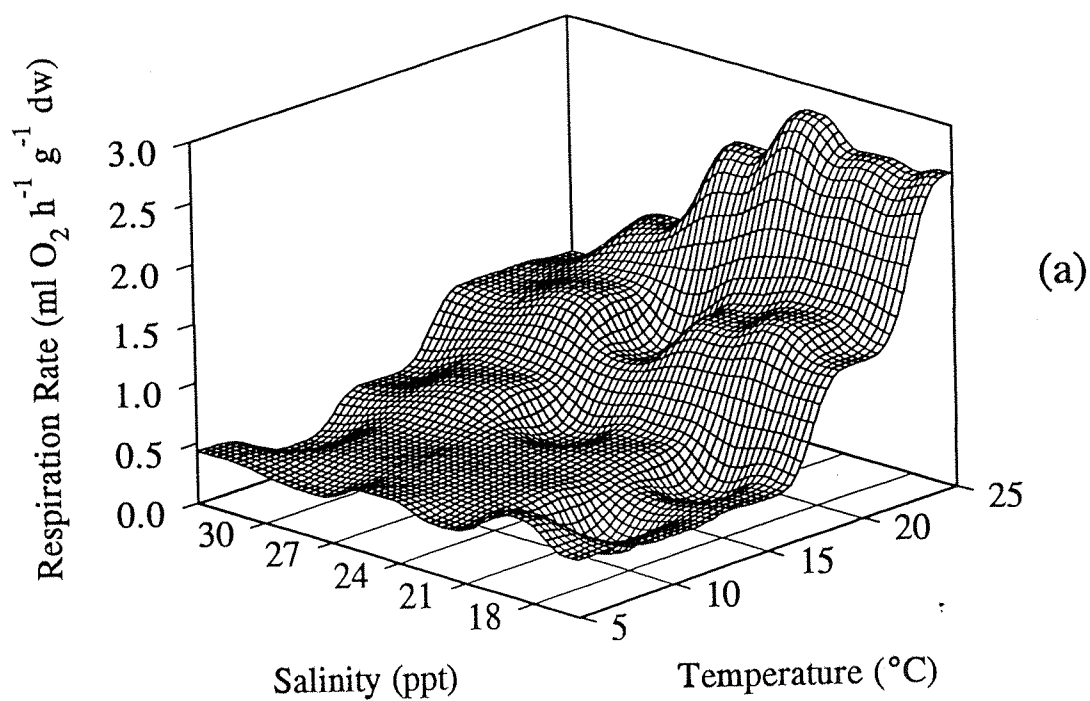


Figure 3.14 Comparison of the effects of temperature and salinity on the respiration rates (ml O₂ h⁻¹ g⁻¹ dry weight) on (a) *Crassostrea gigas* and (b) *Ostrea edulis*. Mean values, $n = 12$ at each point.

dry weight with the maximum rate occurring at 25°C and 22‰ (figure 3.14a). The range of respiration rates for *O. edulis* occurred between 0.06 and 2.00 ml O₂ h⁻¹ g⁻¹ dry weight, with the maximum occurring at 25°C but at a higher salinity (33‰) than that for *C. gigas*. Differences in excretion rates for the two species were also non-significant ($P = 0.530$). High variability was exhibited by both species (figure 3.15) with *C. gigas* having excretion rates in the range 3.65 to 115.33 µg NH₃ h⁻¹ g⁻¹ dry weight, and *O. edulis* a very similar range of 3.52 to 116.91 µg NH₃ h⁻¹ g⁻¹ dry weight. Both species had peaks in excretion rate at low salinities (16-19‰) and high temperatures (20-25°C).

The scope for growth (SFG) values obtained for the two species (figure 3.16) were very significantly different ($P < 0.001$). *O. edulis* actually exhibited negative SFG values (i.e. it was using up energy reserves) with a minimum SFG value of -19.10 J h⁻¹ g⁻¹ dry weight increasing to a maximum of 56.35 J h⁻¹ g⁻¹ dry weight; similar SFG values were obtained across a wide range of conditions between 15°C and 25°C, and 25 to 33‰. Low temperature and low salinity conditions led to negative SFG, and high temperature / low salinity conditions proved lethal. *C. gigas* (figure 3.16a) had a very much wider range of SFG values with no negative values (4.71 - 274.42 J h⁻¹ g⁻¹ dry weight). Those conditions giving optimum SFG appeared to occur at two points, either mid-temperature (15°C) and high salinity (28-33‰) or high temperature (20-25°C) and reduced salinity (22-25‰).

Data were unavailable for body condition index (BCI) in the Solent *O. edulis* so direct comparisons were not possible. The BCI for *C. gigas* is represented in figure 3.17 with optimum conditions occurring at high salinities and a marked decrease at high temperatures and low salinities.

3.3.5 Amoebocytic Effects of Elevated Lactate Concentration

The results of lactate accumulation on the amoebocytes of *O. edulis* are shown in table 3.01. Activity in the cells was observed to cease, with cell remaining aggregated and rounded. Untreated haemolymph showed cells to behave normally, i.e. become flattened and project pseudopodia, before moving apart.

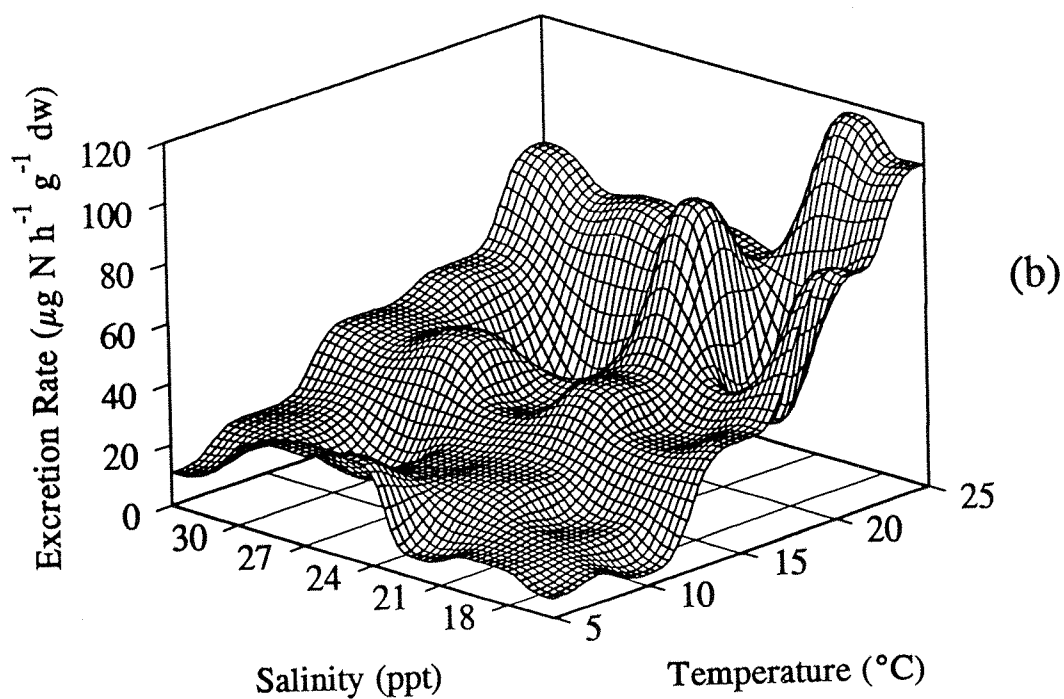
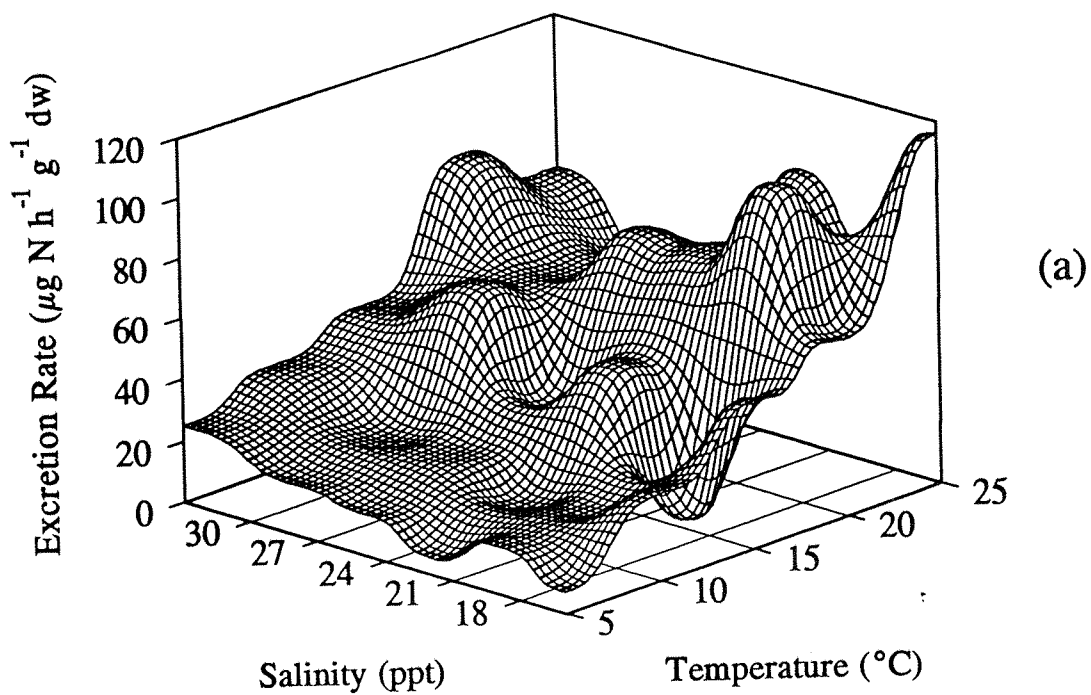


Figure 3.15 Comparison of the effects of temperature and salinity on the excretion rates ($\mu\text{g N h}^{-1} \text{g}^{-1}$ dry weight) of (a) *Crassostrea gigas* and (b) *Ostrea edulis*. Mean values, $n = 12$ at each point.

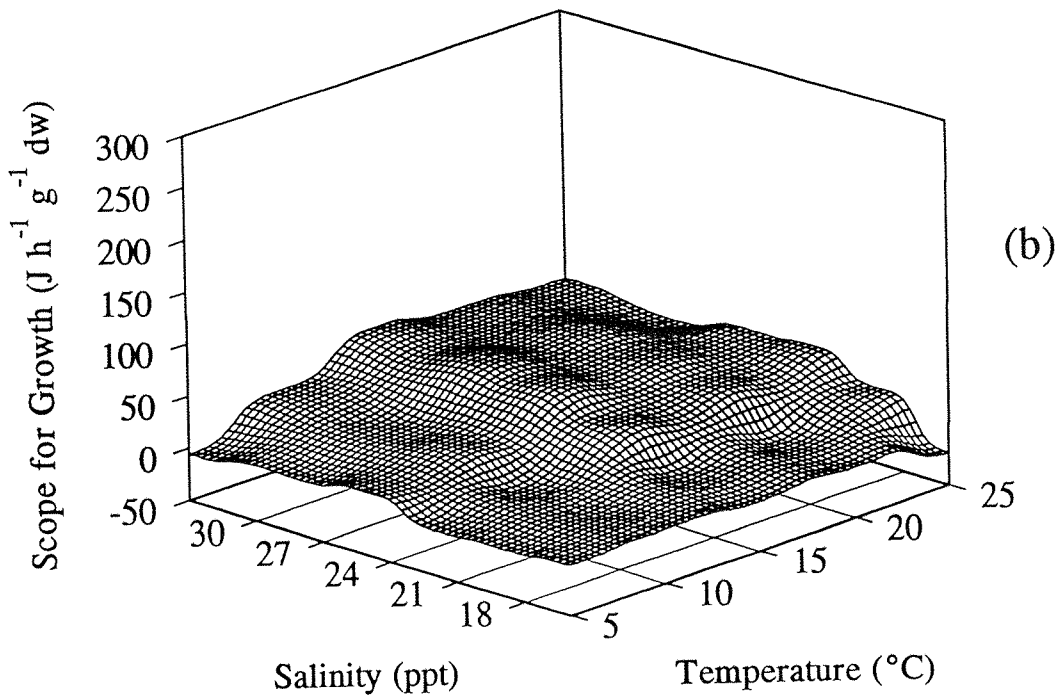
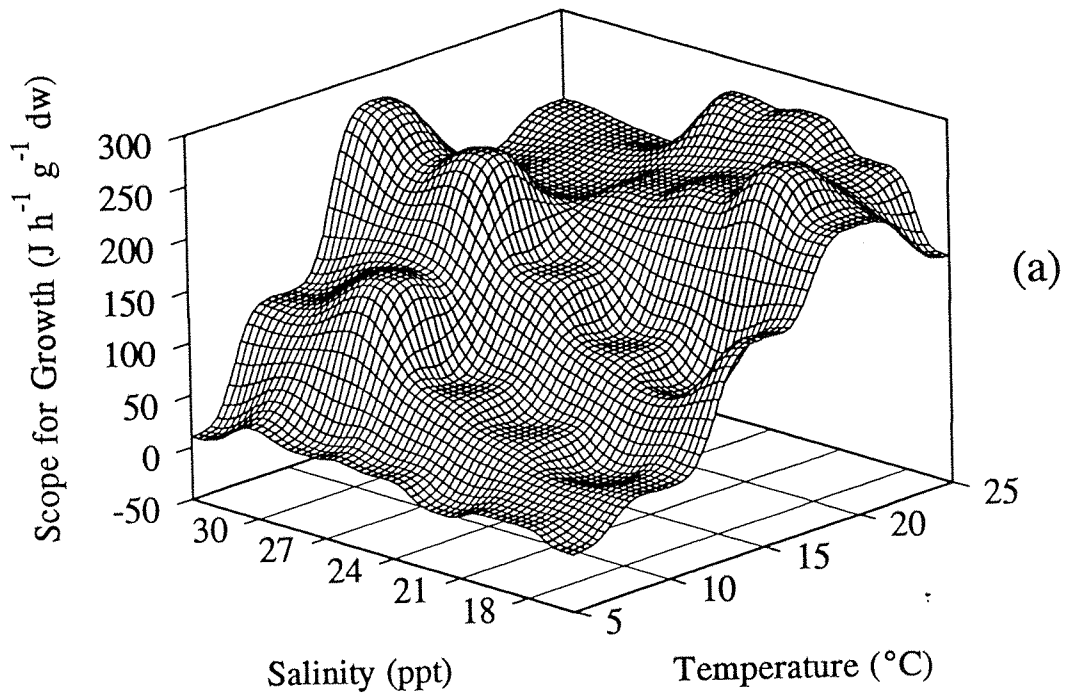


Figure 3.16 Comparison of the effects of temperature and salinity on the scope for growth (J h⁻¹ g⁻¹ dry weight) of (a) *Crassostrea gigas* and (b) *Ostrea edulis*. Mean values, $n = 12$ at each point.

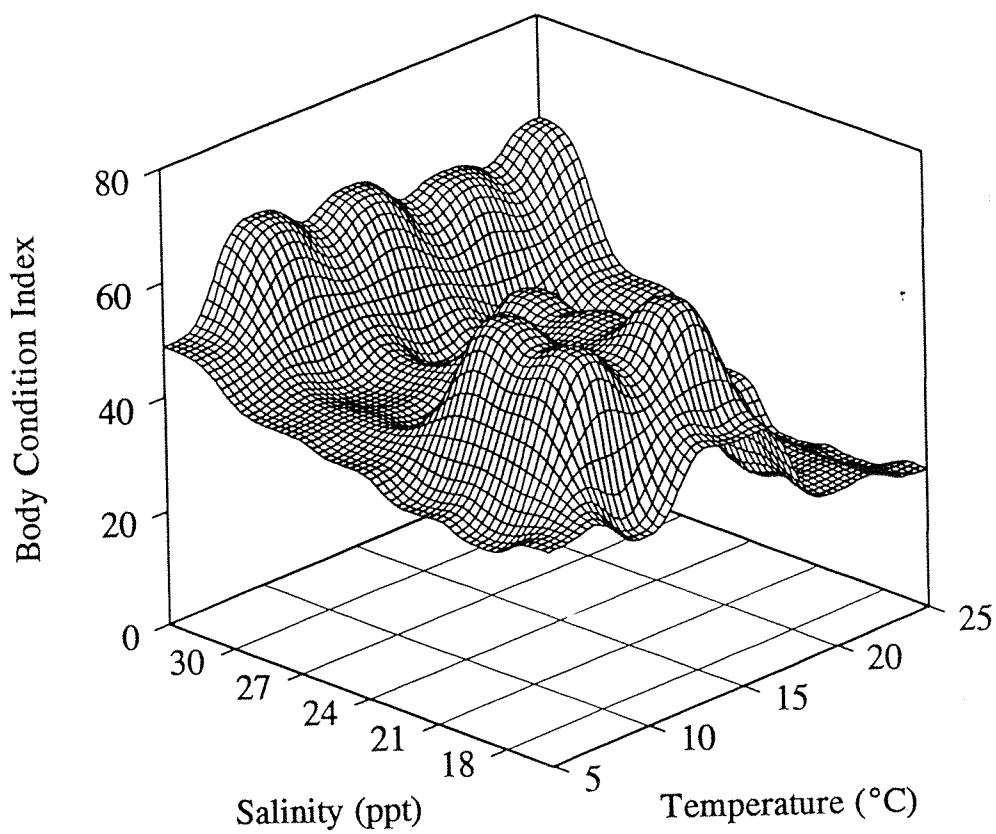


Figure 3.17 Effects of temperature and salinity on the body condition index of *Crassostrea gigas*. Mean values, $n = 12$ at each point.

Observation	Normal Cells	+ Lactate
pH	7.3 - 7.5	7.3 - 7.5
Cell Clumps	Separate	Remain Clumped
Pseudopodia	Present	Disappear after five minutes
Rate of Locomotion ($\mu\text{m min}^{-1}$)	5.34	0
% Size Increase	64	7.41

Table 3.01 Effect of increased haemolymph lactate concentration on large granulocyte (amoebocyte) activity.

3.4 DISCUSSION

3.4.1 Scope for Growth in 3 Populations of *O. edulis*

FILTRATION RATE

It was demonstrated in the previous chapter that filtration rate plays a very important role in determining the energy available for oysters and will ultimately influence how the animals are able to cope with stress and/or disease. It has been shown that a variety of factors influence the rate of food uptake, such as environmental temperature and salinity, food concentration, and animal size. It would have been preferable to use animals of the same size from all populations, but unfortunately the animals used in this study were supplied by the MAFF Fish Diseases Laboratory, and as such there was no choice in the size of animals.

The Solent oysters had lower rates of filtration compared with the other populations and it is this lack of energetic resources for fighting infection which may, hypothetically, have contributed to the mass mortality suffered by these animals. The filtration rates, and other physiological rates, measured showed some overlap between the smaller Loch Sween animals and the larger Conwy oysters. The smallest animals from Conwy had the highest rates of filtration with little difference between the 1992 and 1991 animals of this population. The large variations observed in ingestion rates meant no statistically significant difference was found between the three populations. These differences were not produced as a result of different environmental regimes since all the oysters were collected from, and maintained under, the same conditions. The rates shown by each population are probably a result of a combination of animal size, degree of parasitic infection, and the ecological niche occupied by that population, i.e. animals from different areas cope differently with the same conditions. Although unknown at the time, subsequent investigation showed the degree of parasitic infection to be minimal in the Scottish oysters (Bucke, *pers. comm.*).

The high rates obtained for the smaller animals may well reflect the need for ingested energy to be directed towards somatic growth, which is discussed later in this chapter (section 3.4.3). Larger animals need food largely for maintenance of body tissues and gametogenesis, and less so for somatic tissue production. In the Loch Sween population there was an increase in ingestion rate

in the 1992 animals compared with the 1991 ones, although this was not statistically significant. This increase in filtration rate may contribute to the apparent resistance to *Bonamia* by this population as more food is ingested, supplying more energy to combat the pathogen, for example in the production of microcidal agents by the immunological system. Any drain on the energy resources of the oyster by the parasite, as with *Perkinsus marinus* (Choi *et al.*, 1989) or the MSX parasite *Haplosporidium nelsoni* (Newell, 1985; Barber *et al.*, 1988a; 1988b), would be negated by the additional energy supply. Barber *et al.* (1991) made similar observations to those in this study with *Crassostrea virginica* infected with MSX. Resistant oysters of this species had higher clearance rates in summer than susceptible animals did.

ABSORPTION EFFICIENCY

Absorption efficiency (AE)(figure 3.04), unlike the filtration rate, shows no significant effects of oyster size and thus matches observations made in *Ostrea edulis* by Winter *et al.* (1984). Differences in AE between the populations are probably a result of metabolic processes and not size effects. The higher absorption efficiency observed in Solent compared with Loch Sween animals implies that rate of ingestion does not necessarily dictate the amount of energy absorbed from the food. This is more likely a function of the length of time the food spends in the digestive system. With a faster throughput of seston, the food has a shorter period in the gut leaving less time for absorption and assimilation of energy. The increasing efficiency observed in the Loch Sween population, between 1991 and 1992 animals, may be caused by mild parasite infection compromising the rate of metabolism, resulting in food spending longer in the gut. This extra time for food absorption would be costly to the parasite as it provides more energy for fighting pathogen and it may be this sort of change in metabolism which confers an apparent resistance to bonamiasis. It also enables them to maintain their scope for growth and body condition indices, whilst these decrease in the Conwy population. Seasonal effects resulting from the slightly different sampling times in 1991 and 1992 were probably negligible as the scope for growth measurements for each group were made under the same temperature and salinity conditions, after a

period of acclimatisation to the aquarium system.

RESPIRATION RATES

The amount of oxygen and food available to oyster tissues is determined by the amount of water circulated over the gills. With a significant effect of size on filtration rate it would be expected that size would also influence respiration rate. Figure 3.05b shows that this is indeed the case, with an exponential relationship and a decreasing rate with increasing body size. This relationship had a slightly higher degree of correlation than the filtration rate showed.

Overall, there were no significant differences between the populations because of the high degree of variability observed in the test groups. There was a decrease in the respiration rates between 1991 and 1992 Scottish oysters. This response is the opposite of that seen in MSX resistant and susceptible *C. virginica* oysters (Barber *et al.*, 1991) and may be caused by factors similar to those which affected the absorption efficiency. If metabolic processes are slowed then respiration rate may also become reduced. This uses up less of the animals' energetic resources which are then available for combatting stressful conditions, conferring a degree of resistance to parasitic infection, and the larger tissue weight gives a supply of stored energy enabling them to cope with prolonged stressful periods. The smaller Conwy oysters were less well able to resist infection resulting in increased metabolic demands as the required immunology changed. The variation between individual animals may be attributed to differences in pathogen loads for each animal - numbers of parasites for individual oysters was not determined - resulting in the large errors observed. Although the Solent population had the lowest respiration rates of the three healthy populations it still suffered mass mortality. Unfortunately, the lack of animals resulting from this means it is impossible to determine how respiration was affected by parasitic infection. It may be hypothesised that the disruption of metabolic processes resulted in an increased rate of respiration, more so than in the Conwy population, and this additional burden on energetic resources contributed to the susceptibility and mortality of this population.

NITROGENOUS EXCRETION

There was a considerable, statistically significant difference between the excretion rates of the Loch Sween and Solent animals, and the Conwy population (figure 3.06). This physiological rate proved to have the highest degree of correlation with body size. It is possible that this increase in ammonia excretion is a by-product of other metabolic and physiological processes. The Welsh population showed the greatest filtration rate, and consequently had an increased input of protein, and it is regulation of this protein which resulted in raised nitrogenous output. However, although there was relatively little change in filtration rate between healthy 1991 and the parasitised oysters of 1992, there was a decrease though not statistically significantly so, in excretion. Again, the levels of parasite infection were unknown but any reduction in excretion may have been a response to, or a result of, fighting parasite infection. By reducing the amount of nitrogenous excretion, more amino acids would be available for the production of microcidal enzymes (e.g. lysozyme) or for haemopoiesis. If the oysters have little resistance to the parasite then the decrease in excretion rate may well result from the infection compromising the effectivity of the metabolic processes.

No significant change in excretion rates was observed for Loch Sween animals, although this population did have an increase in filtration rate. With no significant change from the 'normal' rates, these oysters would have no change in energy resources available for stress responses, so raised protein input from increased filtration rates may balance any reduction in metabolic efficiency caused by the parasite.

SCOPE FOR GROWTH

The combination of these physiological measurements gave the scope for growth (SFG) index represented in figure 3.07. As with *Crassostrea gigas* in chapter 2, the similarity between the SFG and filtration rate plots denote that the latter is a major influencing factor in this index. The majority of components of this index showed relationships with body size, and consequently there is a significant, inverse relationship between SFG and dry weight (figure 3.07b). The increase in SFG for the 1992 Loch Sween oysters, compared with the previous

year, reflected the increase in ingestion rate (i.e. an input of energy) and the decrease in respiration rate (a reduced energy output) between the two groups. This 'extra' energy was potentially available for somatic growth or gametogenesis, but it could also have been diverted to the animals' immunological system and other defence mechanisms. If this was the case then the term "scope for growth" is misleading and may be better called scope for disease or stress resistance. Increases in energetic resources in this manner could explain the assumed resistance of this population to bonamiasis.

The decrease in excretion rate for 1992 Conwy animals compared with their healthy stock could not compensate for energy lost through respiration, and consequently this group exhibited a reduced SFG. More importantly these animals had less energy available for coping with the additional stress of parasite infected waters. The two Conwy groups had significantly higher SFG values than the 1991 Loch Sween population, but not when compared other groups or with each other. Again there was a large degree of individual variation, which could have been a reflection of the parasite load in individual oysters. With environmental variables kept constant for all animals studied, the variations observed were mainly caused by size difference. The smaller animals had higher rates compared with the larger animals; this increased rate of intake of water would also increase the chances of ingesting water-borne pathogens, and thus increase the likelihood of parasite infection. It is difficult to establish whether metabolic and physiological modifications were a response of the animals to parasite infection, or whether they were caused by the pathogen directly affecting these processes. However, it did appear that larger oysters were better able to cope with this stress, possibly because of their storage products or because of their different priorities for partitioning energy. It was noted by van Banning (1991) that Dutch oysters only became infected once they reached a certain size and developmental stage. If less energy is directed towards reproductive development or somatic growth, then more can be directed towards disease resistance.

Further evidence provided when other indices of stress assessment, such as adenylate energy charge and immunological measurements were applied to the three populations (Hawkins *et al.*, 1993b), showed that energy partitioning is very

important. Whilst both the Loch Sween and Conwy groups were able to assimilate large amounts of energy, in the Scottish animals the absolute amount of energy devoted to haemocytic and haemolymph processes was much greater than that available for the maintenance of immunocompetence of the Conwy oysters, and presumably the Solent oysters. In these latter two groups most of the energy was diverted to gonadal growth and gametogenesis. This ability of the Scottish population to sustain an active immune response to pathogen challenge meant these animals were the 'fittest', i.e. they could survive and reproduce under the given environmental conditions.

3.4.2 Body Condition Index in Three *O. edulis* Populations

The results of the body condition index (BCI) are shown in figure 3.08. As with the BCI measurements for *Crassostrea gigas* (section 2.4.2) there was an inverse relationship between the SFG index of the individual animals and their BCI. The BCI recorded for the Scottish animals was greater than for the other populations, although the only statistically significant difference was between the two 1992 groups. The increase in BCI between 1991 and 1992 for the Loch Sween oysters was concomitant with a rise in SFG for these animals. As more energy is assimilated and less is lost through respiration, more somatic tissue growth can occur as was reflected in the increased BCI. The diseased Conwy animals had decreased SFG and BCI indices as energy input remained constant but energy loss through respiration was increased. This resulted in less energy being available for somatic growth. Resources are needed to develop the immune system to cope with parasitic infestation since this results in the depletion of stored products and eventually tissue resorption and ultimately causes a decrease in the body condition. These oysters, and the Solent population, became reproductive at a soft tissue weight of 0.2g (Hawkins *et al.*, 1993b) and consequently much of their energy resource was used in gametogenesis. Gauthier *et al.* (1990) showed that animals in reproductive condition are particularly susceptible to parasite infection especially when their gonad index is low. The low BCI observed for 1992 Conwy animals may indicate why these animals are more susceptible to *Bonamia ostreae* infection

than the Scottish oysters, or the decrease could have been a result of the animals already being infected. Parasites have already been shown to have an energetic drain on oyster resources (Newell, 1985; Barber *et al.*, 1988a; 1988b; Choi *et al.*, 1989) so if the Conwy *Ostrea edulis* use up stored reserves to improve the immune system and combat disease infection, then body condition will decrease. BCI and dry weight showed a significant positive, linear relationship which would be expected since dry weight was used in the index calculation.

3.4.3 Energy Partitioning in Three *Ostrea edulis* Populations

It has already been discussed, in section 3.4.1, how differences in physiological rates and absolute energetic requirements vary between the populations, and how these may confer an advantage in resistance to stress or disease. The three populations of *O. edulis* appeared to show distinct differences in the partitioning of their energy even though all animals were kept under the same physico-chemical conditions and were measured before being exposed to the parasite, i.e. they were all healthy animals. The observed differences must therefore have reflected differences in animal size and stage of development.

The Conwy population demonstrated the highest levels of filtration rate (figure 3.03) and SFG (figure 3.07) and when their energy partitioning was examined (figure 3.09) they showed the highest proportion of energy devoted to somatic and reproductive growth. The high degree of individual variability in each population meant that no statistically significant differences appeared when some comparisons were made. However, with this proportion of assimilated energy directed into SFG, it is perhaps surprising why this population was as susceptible as it was to *B. ostreae*. Other studies have shown that the parasite *Perkinsus marinus* may consume 5% of the SFG energy budget of *C. virginica* under moderately heavy infections (Choi *et al.*, 1989). Even if this percentage of the energy budget is lost to parasite infection, it would be expected that the remaining allocation of assimilated energy would cope with the extra energetic burden. Unfortunately this energetic drain is persistent and requires that the oyster is consistently ingesting enough food to supply the required energy, so if the oyster

is already committing a high percentage of its SFG budget to gametogenesis and reproduction, much less energy is then available for immunological processes and fighting disease. Similar proportions of *Tapes philippinarum* and *Crassostrea gigas* energy budgets were dedicated to SFG but these animals are not susceptible to *B. ostreae* and consequently this did not affect their health. An interesting comparison would be to incubate these species with pathogens to which they are sensitive, such as *Perkinsus marinus* (Brown Ring disease in clams) or *Microcytos mackini*, causing Denman Island disease in *C. gigas* (Farley *et al.*, 1988) and see what modifications, if any, are made to the way in which they partition energy.

Loch Sween animals had the lowest absolute SFG index (figure 3.07) and showed the lowest percentage of energy budget directed to SFG (28% of the total ingested energy, figure 3.11). This was significantly lower than the other oyster populations, but when other metabolic processes of respiration and nitrogenous excretion were taken into account, it was comparable with species such as *Ostrea chilensis* which uses 58% of the energy ingested for routine metabolic maintenance (Winter *et al.*, 1984). Although this population had less energy devoted to SFG it still had an apparent degree of resistance. This was probably a result of the larger size and later stage of development of these animals when compared with the other more susceptible populations. With less of the energy budget allocated for reproductive purposes, and more directed towards somatic processes, these oysters were better able to cope with stressful conditions. A lowering of metabolic rates resulted in seston spending a longer period in the gut of the Scottish oysters and consequently they show a high absorption efficiency. However, the partitioning of the energy showed that more than half the ingested energy was never assimilated. This further suggests that it is the fate of the assimilated energy, whether for somatic or gametogenic purposes, which determines any resistance to stress and not the absolute amount of energy available (Hawkins *et al.*, 1993c). The energy used through respiration was significantly higher in this group than the others but there was an increase in respiration rates with increasing size of oysters. This is concordant with data presented by White *et al.* (1988a; 1988b) whereby respiration makes up a smaller fraction of the total energy budget in smaller oysters.

The data presented for energy partitioning for the different populations and

different species supports the findings of the physiological measurements. Many of the differences appeared to be size related or were a factor of the stage of reproductive development. This determined how energy available for SFG was utilised, whether for somatic growth or gametogenesis. The actual amount of energy potentially available appeared to have little bearing on the animals' resistance. Similar proportions in energy partitioning have been demonstrated for the different species but whether this confers an advantage in stress or disease resistance can only be determined with trials of pathogens virulent to these species (Hawkins *et al.*, 1993c). The percentage of the ingested energy allocated to each metabolic process may change under different environmental conditions, resulting in oysters demonstrating different disease resistance in a changing environment.

3.4.4 Comparative Physiology of *O. edulis* and *C. gigas*

One of the major aims of this study was to compare the physiology of different species of bivalves of commercial interest. Chapter 2 provided comprehensive measurements of *Crassostrea gigas* physiology across a matrix of temperature and salinity and in this chapter the native species of oyster, *Ostrea edulis*, was introduced, and how its physiology varies between populations and under pathogen load. The introduction of *C. gigas* as a commercial replacement for the diseased flat oyster made it desirable to compare directly their physiology under the same conditions. This would indicate areas in which one species may be more suited than the other, and by extrapolating these data to the natural environment, predictions could be made of which species would adapt best to the prevailing conditions. The use of three-dimensional surface plots make these comparisons easily possible.

Figure 3.12 shows a dramatic difference in the range of filtration rates for both species. *C. gigas* had a much wider range showing highest rates at high temperature but reduced salinity. Under these conditions *O. edulis* was found to suffer severely and more than one week under this regime resulted in large mortality. The optimal conditions for the native species occurred at high temperatures and high salinities indicating its preferred marine physiology.

Although the animals used for this study were similar in size, the differences in observations were too great to be a result of weight differences. Animals were treated and maintained in the same fashion with identical food rations so the differences in filtration rate therefore reflected the natural ability of the *Crassostrea gigas* to filter large quantities of water. The absorption efficiency of the two species (figure 3.13) also proved to be significantly different, although they demonstrated similar ranges.

Respiration rates (figure 3.14) for both species showed similar ranges but under different conditions. As with the filtration rate, *O. edulis* showed greatest respiration at high temperature and high salinity. These rates were shown to increase linearly between 10°C and 25°C with a discontinuity below 10°C. This difference was attributed to a change between 'winter' and 'summer' physiological states, a trait also demonstrated by *C. gigas* with a linear increase at higher temperatures but the discontinuity marking the different seasonal physiologies at slightly higher temperature. This difference between the species is important as it indicates the different seasonal ranges of temperature normally endured by these animals. The more tropical species would usually have warmer winter conditions giving rise to a winter physiology beginning at a higher temperature compared with the cooler waters of the temperate species. The optimal conditions for respiration in *C. gigas* were again indicative of a preferred warmer, estuarine regime. Both species demonstrated a high variability in excretion rates so no significant difference was observed when they were compared (figure 3.15). Similar ranges were demonstrated with a general increase in excretion rate with temperature, as well as maximum ammonia excretion occurring under similar conditions.

The scope for growth (SFG) of the two species was found to be significantly different (figure 3.16), predominantly as a result of the high filtration rate in *C. gigas*. At low temperatures, and to a lesser extent at low salinity, *Crassostrea* was able to maintain basic metabolic processes without having to use stored reserves. The high filtration rates (figure 3.13) lead to large amounts of energy being assimilated and resulted in the high SFG recorded. This demonstrates a high degree of metabolic and physiological plasticity which could be utilised by

the oyster to survive in a wide range of conditions and thus makes *C. gigas* a highly desirable commercial species. *Ostrea edulis*, however, had a limited SFG owing to its low range of filtration rates. At low temperature and low salinity negative SFG was observed causing these animals to use up stored reserves (resulting in decreased body condition and a lack of gametogenesis). A combination of high temperature and low salinity proved lethal to *O. edulis* and consequently narrowed the range of environmental conditions under which this species can survive. Optimum SFG occurred at high salinity confirming its more marine orientated physiology. The deleterious effects of certain combinations of environmental variables may occur in the natural environment, for example with high rainfall or river runoff in summer, and probably increases the disease susceptibility of *O. edulis*. Any increase in physiological stress can lead to massive mortality within populations so the wide range of conditions tolerated by *C. gigas*, coupled with its high SFG, make this species an ideal commercial replacement for the flat oyster.

Unfortunately no data were available for body condition index (BCI) in *O. edulis* but the BCI of *C. gigas* (figure 3.17) showed conditions under which this species had directed a lot of energy into visceral production rather than shell growth. This is useful information for conditions under which to fatten oysters for the commercial market, but may also give an indication of conditions under which the gonads ripen, i.e. the best conditions for spawning oysters.

3.4.5 Lactic Acid Effects on Haemocyte Activity

The phagocytic large granulocytes which circulate in the haemolymph permeate connective tissue and lie on the luminal surfaces of digestive epithelia. Any digestible invading pathogens are destroyed in phagosomes and those which may be resistant to intracellular digestion are removed by diapedesis through epithelial membranes. The energy requirements for this are supplied by ATP derived from glycolysis and so are independent of aerobic metabolism. In *Mercenaria mercenaria* haemocytes, treatment with potassium cyanide fails to alter particle uptake (Cheng, 1976). Thus, if bivalves become exposed during low tides,

the resulting anaerobia would not be detrimental to the immunocompetence of the animals. Under these circumstances, the Ostreidae accumulate succinate in preference to the more toxic lactate (Hammen, 1969; Mustafa and Hochachka, 1971; Smyth, 1976). This accumulated succinic acid can be used by haemocytes to sustain their activities, but prolonged anoxia results in lactate production. Chronic tidal exposure has been shown to affect haemocyte mediated defences of *M. mercenaria*, especially the production of hydrogen peroxide (Hawkins *et al.*, 1993a). With haemocyte viability, size, and phagocytic activity unaffected by oxygen tension (Alvarez *et al.*, 1992), this study examined the effects of accumulated lactic acid on large granulocyte function.

The results presented in table 3.01 indicated that the presence of lactate has a significant effect on haemocyte activity. Although the acid levels had increased, the medium maintained a constant pH range for both control and experimental groups; the influence of pH was therefore eliminated from the investigation. Locomotion of cells was inhibited by lactate presence, causing cells to remain clumped and rounded - any change in cell size was negligible. The way in which lactate inhibits this movement is unknown but it may be directly toxic to microtubule formation, and consequently the production of pseudopodia and thus locomotion, or its effects may be indirect acting on other metabolic pathways in the cell. The lack of activity observed in these cells would also explain some of the observations made by Alvarez *et al.* (1992). They noted that although anaerobiosis did not affect haemocyte viability there was a ten-fold decrease in circulating haemocyte concentration. They were unable to ascertain whether this resulted from inhibited haemopoiesis or whether there was a lower level of mobilisation of circulating haemocytes. The results of this experiment with increased lactate suggest that it may be a combination of both, with haemocyte activity inhibited and the toxic nature of lactic acid reducing the metabolic process of haemocyte production.

3.4.6 Summary

The comparison of physiology in three *Ostrea edulis* populations showed

there to be a high degree of variability in many of the observations made, which was probably a function of the numbers of parasites infecting individual animals. Solent *O. edulis* demonstrated the highest levels of mortality with no live animals left at the 1992 sampling. Oysters from Scotland were the largest of the three populations and showed the lowest mortality. Their increase in energy input and decrease in energy expenditure, between 1991 and 1992, ensured an increased scope for growth (SFG) allowing this population to cope with any infection. The Conwy oysters showed increased respiration and decreased SFG, reflected by the increased mortalities. The body condition index measured for each population was inversely proportional to the SFG, as in *Crassostrea gigas* (chapter 2) and again was probably a reflection of reproductive condition, and thus a time at which most animals become increasingly susceptible to disease.

When the energy partitioning was examined, all the populations from Conwy (*O. edulis*, *C. gigas* and *T. philippinarum*, which is discussed in the next chapter) showed similar proportions of the energy budget directed to each process. The high proportions of energy put into SFG by Conwy oysters was a function of their small size - the larger Scottish oysters put significantly less energy into SFG although this amount was comparable with other *Ostrea* species. The apparent resistance of this population suggests that it is the fate of assimilated energy, whether put into somatic or reproductive processes, which enhances resistance and not the absolute amount of energy available.

Comparison of physiology between *C. gigas* and *O. edulis* showed that the major difference between them was the wide range of filtration rates demonstrated by the Pacific oyster, resulting in *C. gigas* having much higher SFG values. Different optimum conditions were illustrated, especially by respiration rates, with *O. edulis* optima at 33‰/20°C and *C. gigas* at 20-25‰/25°C. A change between winter and summer physiologies was also shown with the *Ostrea* change at 10-12°C and *Crassostrea* at 15°C.

Haemocytic indices showed little significant variation with temperature although other studies have found greater variation with salinity changes. Lactic acid had a highly significant on haemocyte activity, inhibiting it and possibly being toxic to haemopoiesis.

CHAPTER FOUR

PHYSIOLOGICAL AND IMMUNOLOGICAL MEASUREMENTS IN THE MANILA CLAM

Tapes philippinarum

CHAPTER 4

PHYSIOLOGICAL AND IMMUNOLOGICAL MEASUREMENTS IN THE MANILA CLAM

Tapes philippinarum

4.1 INTRODUCTION

Tapes philippinarum were introduced to the study for the sake of a broader perspective in comparing commercially important bivalve species. Physiological and immunological measurements were made, although over a more limited range of environmental conditions. Although no baseline immunological responses have been reported in this study for the two oyster species, they are described by Hawkins and Hutchinson (1990) and Hawkins *et al.* (1993b).

4.1.1 The Use of *Tapes philippinarum* as a Commercial Species

The successful introduction of *Crassostrea gigas* and *Mercenaria mercenaria* into Europe, together with the development of hatchery and aquaculture techniques, encouraged the importation of the Manila clam *Tapes philippinarum*. The first introductions of this bivalve were in France in 1973, from Puget Sound, Canada (Flassch and Leborgne, 1992) with further imports into Spain and Italy in the 1980s via stock from Britain.

T. philippinarum naturally exists between the latitudes 25° and 45° N and was accidentally imported into North America with Pacific oysters from Japan, in 1936 (Quayle, 1964). Since 1941 Manila clams have colonized the coastline from Vancouver to California. The initial research on this clam investigated how it adapted to the temperate Atlantic coastline, as well as developing production control techniques. Further work has examined reproductive biology (Beninger and Lucas, 1984; Ponurovsky and Yakovlev, 1992), nutrition and diet (Laing and Millican, 1991; Laing, 1993), and the continued refinement of hatchery culture

(Utting and Spencer, 1991).

The introduction of novel species brings the added problems of new epizootic diseases. Manila clams have been shown to be resistant to many pathogens but have suffered two major outbreaks of disease in France. In 1987 an outbreak in hatchery stocks was anonymously described and was isolated as a *Vibrio* bacterial strain, labelled as *Vibrio* of *Tapes* (= *Ruditapes*) *philippinarum* (VRP). Sanitary procedures prevented the spread of this disease but a second and potentially more devastating outbreak occurred in the natural environment in 1987. This disease, Brown Ring Disease, affected both spat and juvenile clams and was diagnosed as being typical of *Vibrio* spp. (labelled *Vibrio* P1); treatment for this problem has mainly been by antibiotics (Noel, 1991).

There is a large market for Manila clams especially in Italy, Spain and the far east. This market is being supplied more and more from natural stocks, e.g. 6000 tonnes produced in the Venice lagoon in 1989 compared with 500 tonnes from aquaculture techniques in Brittany in 1990 (Flassch and Leborgne, 1992). Overall, European production of *T. philippinarum* had grown to 20000 tonnes annually by 1990.

4.1.2 Introduction of *Tapes philippinarum* into Britain

The Manila clam was introduced into Britain from broodstock in the State of Washington, USA in 1980 (Utting and Spencer, 1992). As a hardy, fast growing species with a very valuable commercial market, it was attractive for the farming industry. The original fifty clams imported were used to build up hatchery stock at the MAFF laboratory, Conwy and it was these clams ultimately supplied Spain and Italy. Current production in the U.K. stands at only 5 tonnes per year and this is sustained with hatchery produced seed.

Investigations are being made into further control of the breeding and recruitment of these clams, for example by inducing polyploidy with cytochalasin-B (Allen *et al.*, 1989). This sort of control can lead to animals of larger size and different quality. Selective breeding may also lead to disease resistance in clams, maximising the value of the crop. A genetic survey on French stocks (Moraga,

1986) showed that this species is highly heterozygotic which could explain its ability to adapt to temperate waters. However, the quantification of physiological responses to different environmental conditions is necessary to provide information on how infection or disease affects these animals. The use of additional immunological responses, under different conditions, provides more comprehensive baseline levels for the animals, and allows infection of clams to be investigated at several levels. With the application of this suite of methods to many different bivalve species, comparative data can be acquired and optimal conditions for the rearing of each animal determined. Information at this level for *T. philippinarum* is limited (e.g. Goulletquer *et al.*, 1983) and so this study has included the Manila clam in its investigations.

4.2 MATERIALS AND METHODS

4.2.1 Collection and Maintenance of Clams

Tapes philippinarum of approximately 15-25g wet shell weight were provided by MAFF, Conwy. These animals were maintained in flow-through tanks at ambient salinity (31-33‰) and temperature (13-19°C) conditions and were fed with the same algal diet as previously described. Acclimation to the experimental conditions was performed slowly over a period of 48 hours, under micro-processor control. Scope for growth parameters were measure as described previously, for a range of temperatures (5-25°C) at one salinity only (33‰).

4.2.2 Haemocytic Indices in *Tapes philippinarum*

Samples of haemolymph were taken from the adductor muscle sinus by aspiration with a drawn-out, acid cleaned Pasteur pipette and placed in a covered embryo dish previously coated with silicones using Repelcote™.

In vitro measurements of live haemocytes were made using a calibrated video microscopy system in conjunction with Nomarski differential contrast interference optics to visualise the cells. 50 µl of haemolymph was diluted with an equal volume of seawater at the acclimation temperature and placed on a 'Neubauer' haemocytometer. The dilution with seawater facilitated cell counting and promoted the initial aggregation of haemocytes, without the introduction of potential artifacts caused by the manipulation of calcium, and other divalent cations in the diluent, to control cell movement as used in other methods described by Fisher and Newell (1986). Haemocytes were identified and classified into three types according to a scheme adapted from that proposed by Auffret (1988) for *Ostrea edulis* and equivalent to that used by Chagot (1989):

Hyalinocytes - small, dense, agranular and spherical cells 2-4 µm diameter, producing hydrogen peroxide on contact with foreign particles by 'bursts' of respiratory activity.

Small Granulocytes - spherical cells containing numerous dense granules and 8-10 μm diameter.

Amoebocytes (= Large Granulocytes) - irregularly shaped, motile cells often containing a small number of granules and when aggregated 10-15 μm in diameter but capable of producing pseudopodia 2 to 3 times this size and engulfing foreign material.

Video recordings were made of the dispersal of amoebocyte aggregations and subsequent examination of these recordings, using JAVA™ image analysis software, permitted measurements of cell dimensions and rates of locomotion of individual cells following dispersion of aggregates, as well as counts of the three cell types. Rates of locomotion and percentage cell size change were used as indices of haemocyte activity and potential efficiency in removal of foreign material, following the methods described by Fisher and Newell (1986).

4.2.3 Statistical Analysis of Results

The results of laboratory experiments were expressed as mean values \pm standard error, unless otherwise stated. The number of observations at each datum point for the scope for growth measurements was 12 with the haemocytic indices measured for $n=6$ at each datum point. Where appropriate data were analysed using an unpaired t-test (or a Mann-Whitney test as non-parametric alternative) for comparing two conditions, or a standard ANOVA (or a Kruskal-Wallis test as a non-parametric alternative) for comparing more than two conditions. Pearson product-moment correlations were performed to measure the association between variables; this test assumed the data were normally distributed and did not assign dependent and independent variables.

4.3 RESULTS

4.3.1 Scope for Growth in *Tapes philippinarum*

The filtration rates for *T. philippinarum* at a range of temperatures are shown in figure 4.01. These animals were supplied with algae at a particulate concentration of 25,000 cells per cm² as with the oysters. A Kruskal-Wallis one-way ANOVA showed significant differences occurred between low temperatures (5 & 10°C) and 15°C ($P < 0.05$), and a significant increase between 15°C and 25°C ($P < 0.05$). There was no significant difference between 15°C & 20°C or between 20°C & 25°C ($P > 0.05$). The absorption efficiencies are shown in figure 4.02 and range from 0.74 ± 0.04 to 0.85 ± 0.10 , but there was no significant difference between any of the temperatures ($P > 0.05$).

Significant differences in respiration rate (figure 4.03) occurred between the low temperatures (5°C & 10°C) and all the higher temperatures ($P < 0.05$). There was no significant difference between 15°C & 20°C, 15°C & 25°C, and 20°C & 25°C, or between 5°C & 10°C ($P > 0.05$). The maximum range of respiration rates was 0.22 ± 0.07 ml O₂ h⁻¹ g⁻¹ dry weight to 0.62 ml O₂ h⁻¹ g⁻¹ dry weight at 25°C. Excretion rates are shown in figure 4.04. There was no significant difference between 10°C & 15°C, and between 20°C & 25°C ($P > 0.05$). All other comparisons proved to be significantly different ($P < 0.05$).

These physiological measurements were used for the scope for growth (SFG) index shown in figure 4.05. As in the previous figure there was no significant difference between 15°C & 20°C, and also between 5°C & 10°C ($P > 0.05$) but when individual components were compared, using a Pearson product-moment correlation (table 4.01), it was found that filtration rate has a highly significant effect on SFG ($r = 0.960$; $P < 0.001$). Excretion rate also had a significant effect, although the correlation with SFG was not as strong ($r = 0.458$; $P < 0.001$). Respiration rate and SFG had no significant correlation ($r = 0.254$; $P > 0.05$). Filtration rate and excretion rate tended to increase together ($r = 0.435$; $P = 0.001$).

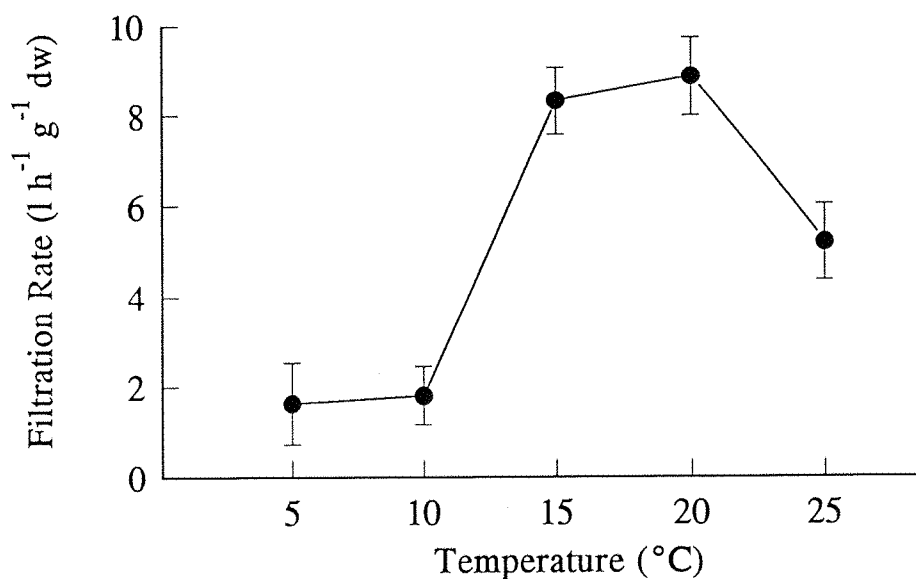


Figure 4.01 The effect of temperature on the filtration rate ($\text{l h}^{-1} \text{g}^{-1}$ dry weight) of *Tapes philippinarum* at 33‰. Mean \pm SE, $n = 12$ at each point.

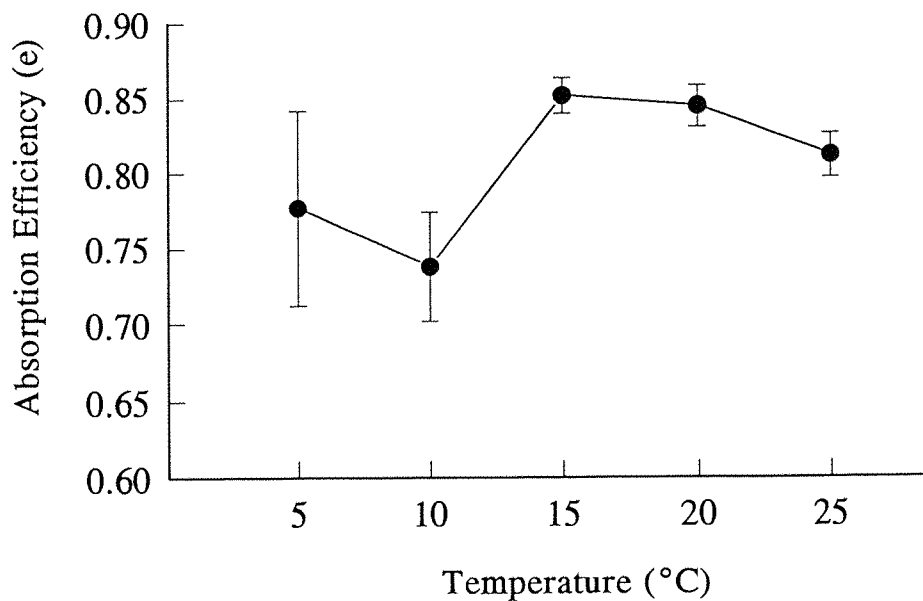


Figure 4.02 The effect of temperature on the absorption efficiency (e) of *Tapes philippinarum* at 33‰. Mean \pm SE, $n = 12$ at each point.

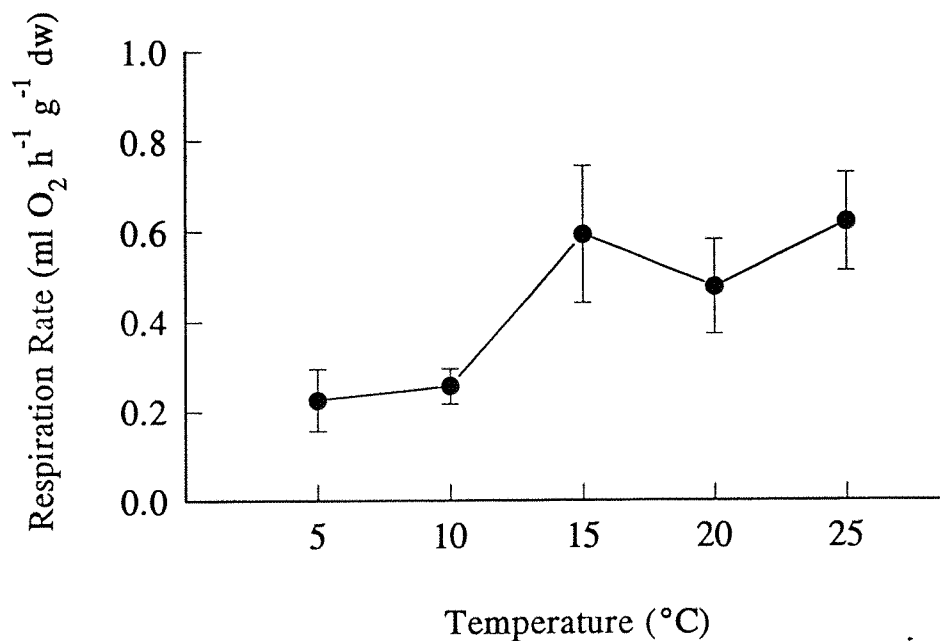


Figure 4.03 The effect of temperature on the respiration rate (ml O₂ h⁻¹ g⁻¹ dry weight) of *Tapes philippinarum* at 33‰. Mean ± SE, n = 12 at each point.

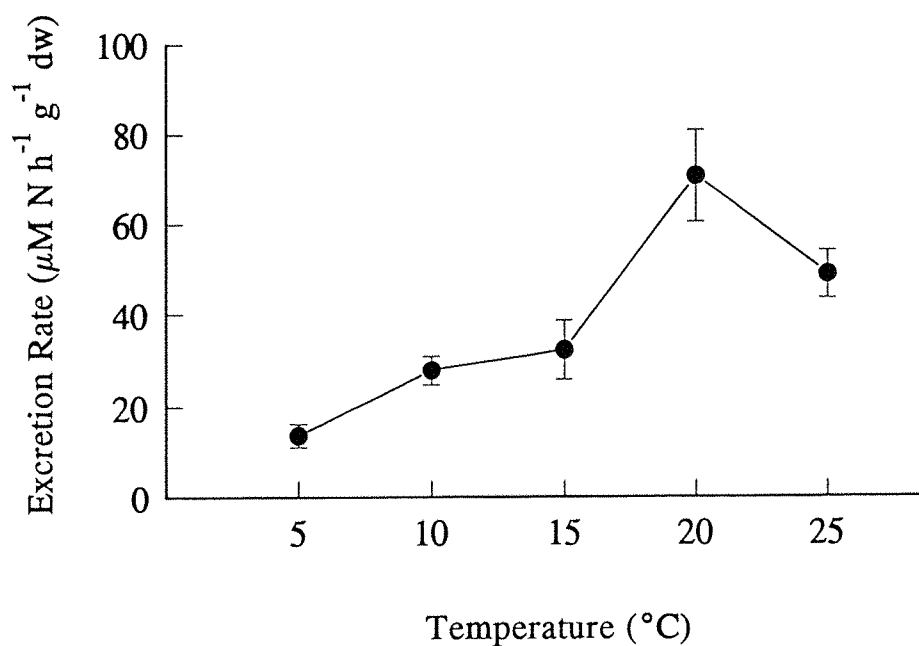


Figure 4.04 The effect of temperature on the excretion rates (μg N h⁻¹ g⁻¹ dry weight) of *Tapes philippinarum* at 33‰. Mean ± SE, n = 12 at each point.

	BCI	Filtration	Respiration	Excretion
SFG	-0.435 0.001 54	0.960 < 0.001 54	0.254 0.063 54	0.458 < 0.001 54
BCI		-0.365 0.007 54	-0.219 0.111 54	-0.241 0.080 54
Filtration			0.288 0.035 54	0.435 0.001 54
Respiration				0.101 0.465 54

Table 4.01 Pearson product moment correlation for physiological components of *T. philippinarum*. Cell contents: Correlation Coefficient, *P* Value, Number of Samples. The pairs of variables with positive correlation coefficients and *P* values below 0.050 tend to increase together. For the pairs with negative correlation coefficients and *P* values below 0.050 one variable tends to decrease while the other increases. For pairs with *P* values greater than 0.050, there is no significant relationship between the two variables.

4.3.2 Body Condition Index in *Tapes philippinarum*

Figure 4.06 shows the body condition index (BCI) in *T. philippinarum* between 5°C & 25°C. There was a significant decrease from 10°C to 15°C ($P < 0.05$), and the subsequent increase from 15°C to 25°C was also significant ($P < 0.05$). The other comparisons were not statistically significant different ($P > 0.05$). The graph appears to show an inverse pattern to that of SFG (figure 4.05), and when a Pearson product-moment correlation was performed a highly significant correlation was found for the two indices ($r = -0.435$; $P = 0.001$).

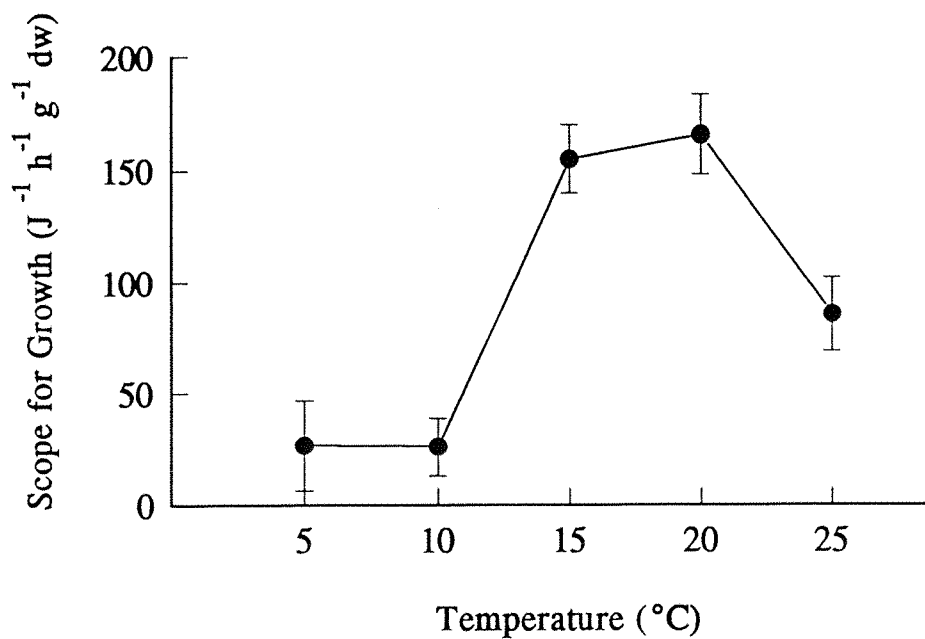


Figure 4.05 The effect of temperature on the scope for growth ($\text{J h}^{-1} \text{g}^{-1}$ dry weight) of *Tapes philippinarum* at 33‰. Mean \pm SE, n = 12 at each point.

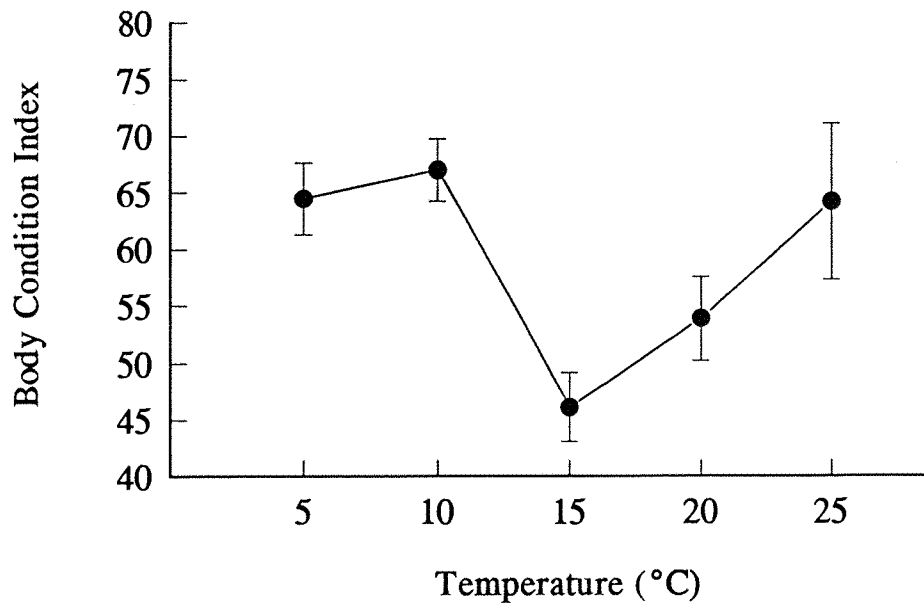


Figure 4.06 The effect of temperature on the body condition index of *Tapes philippinarum* at 33‰. Mean \pm SE, n = 12 at each point.

4.3.3 Energy Partitioning in *Tapes philippinarum*

The relationships between energy consumed, assimilated and expended, and the resulting scope for growth, are represented in figure 4.07. The fate of consumed energy is shown in figure 4.08. *T. philippinarum* showed significantly different energy input into respiration when compared with all the oyster populations measured ($P < 0.05$). Energy lost through ammonia excretion was significantly different from Conwy and Loch Sween oysters ($P < 0.05$) but not from *C. gigas*, and energy lost as faeces and pseudofaeces was significantly different from *C. gigas* and Loch Sween animals ($P < 0.05$), but not from Conwy oysters. The proportion of energy available for SFG was only significantly different from the Loch Sween animals ($P < 0.05$).

4.3.4 Haemocytic Indices in *Tapes philippinarum*

The results of total haemocyte counts are shown in figure 4.09. A Kruskal-Wallis test indicated that there was a statistically significant difference between the median values ($P = 0.034$), but pairwise multiple comparisons of each of the temperatures indicated no significant differences, probably because of the small sample size. Mean haemocyte numbers were in the range 1.20×10^6 to 2.87×10^6 cells per ml. There were no significant differences in amoebocyte numbers (figure 4.10) between any of the temperatures investigated. These cell numbers were in the range 2.02×10^5 to 3.6×10^5 cells per ml.

Small granulocyte numbers are represented in figure 4.11. These numbers increased significantly between 5° & 10°C and 5° & 20°C ($P < 0.05$). There was no significant difference between the other temperatures. Hyalinocytes represented the largest fraction of the haemocyte population in the haemolymph with numbers an order of magnitude larger than for either small or large granulocytes (figure 4.12). There was a statistically significant decrease in cell numbers between 5° & 25°C ($P < 0.05$) but not between the other combinations of temperatures.

Hyalinocyte diameter and amoebocyte initial diameter (figures 4.13 and 4.14 respectively) showed no significant differences at all, over the range of

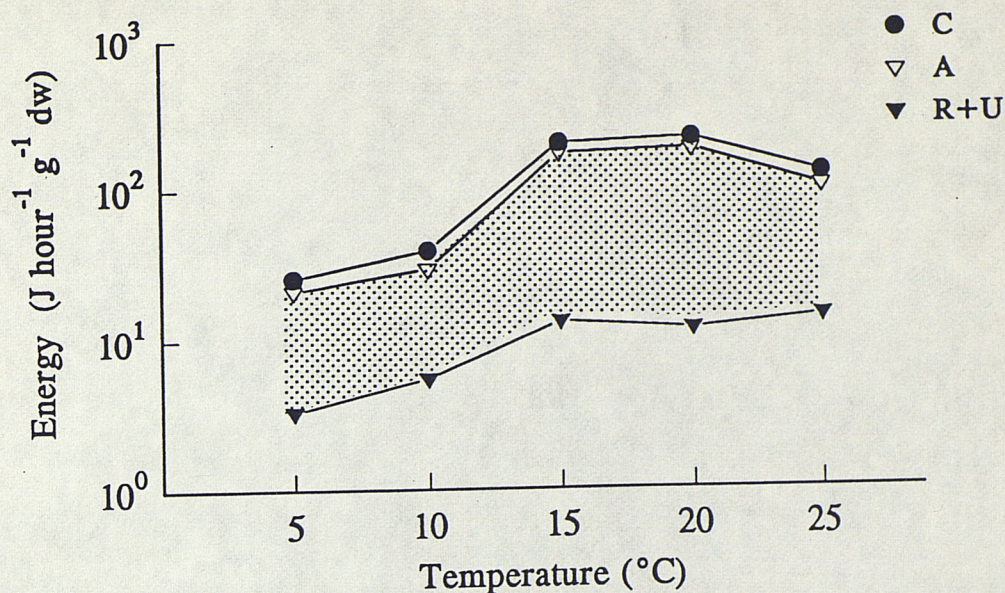


Figure 4.07 The effect of temperature on scope for growth (shaded area) and components of the energy equation, in *Tapes philippinarum* at 33‰. Mean values, $n = 12$ at each point.

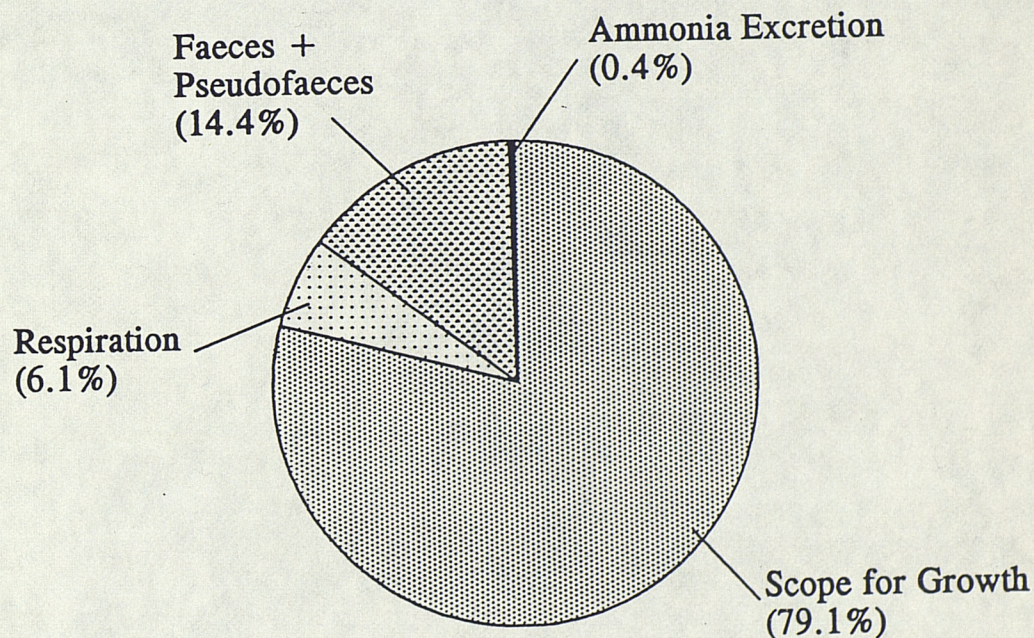


Figure 4.08 Partitioning of ingested energy in *Tapes philippinarum* at 15°C and 33‰. Mean values, $n = 12$.

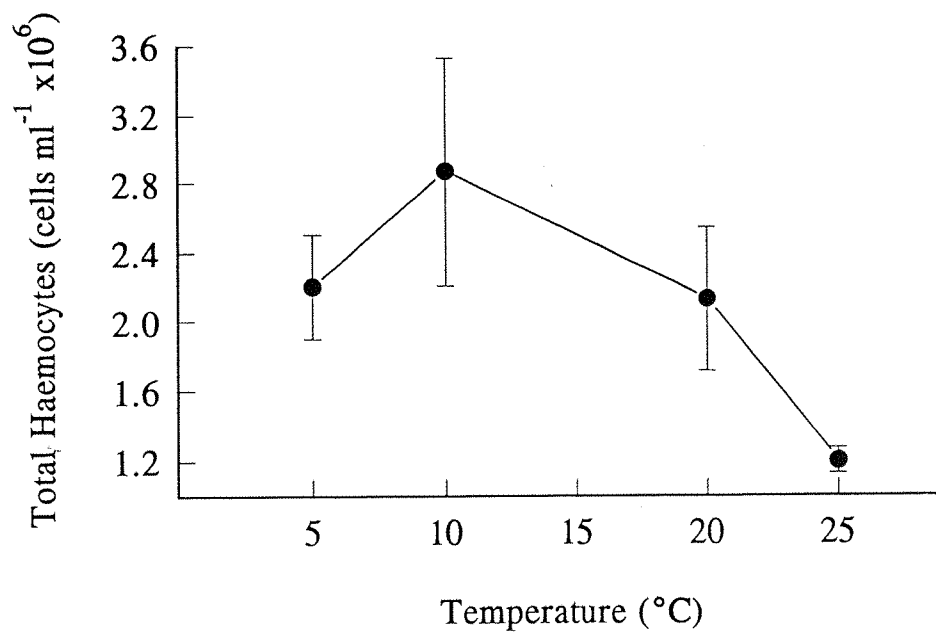


Figure 4.09 The effect of temperature on total haemocyte numbers (cells ml⁻¹ x 10⁶) of *Tapes philippinarum* at 33‰. Mean \pm SE, n = 12 at each point.

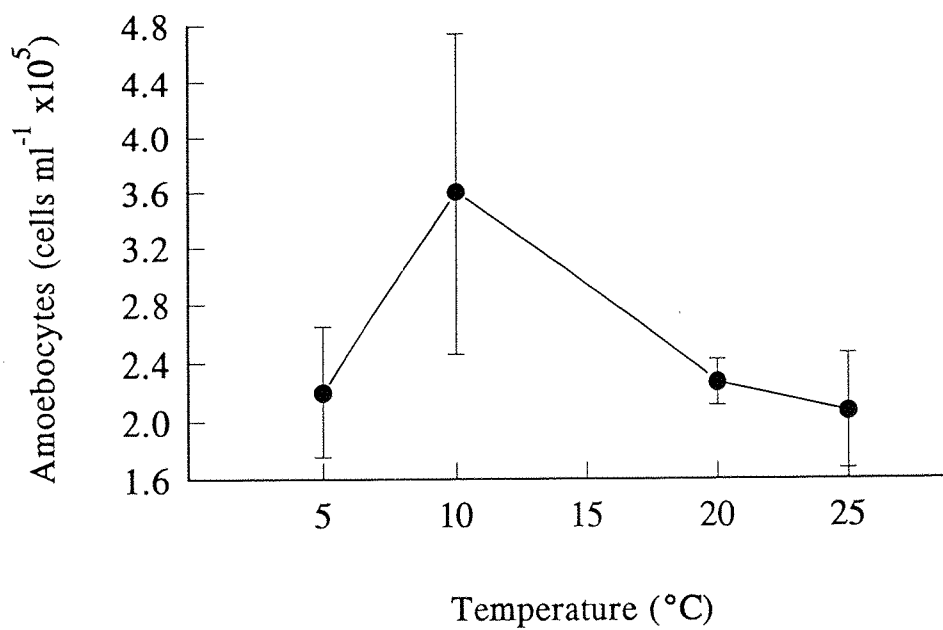


Figure 4.10 The effect of temperature on amoebocyte numbers (cells ml⁻¹ x 10⁵) of *Tapes philippinarum* at 33‰. Mean \pm SE, n = 12 at each point.

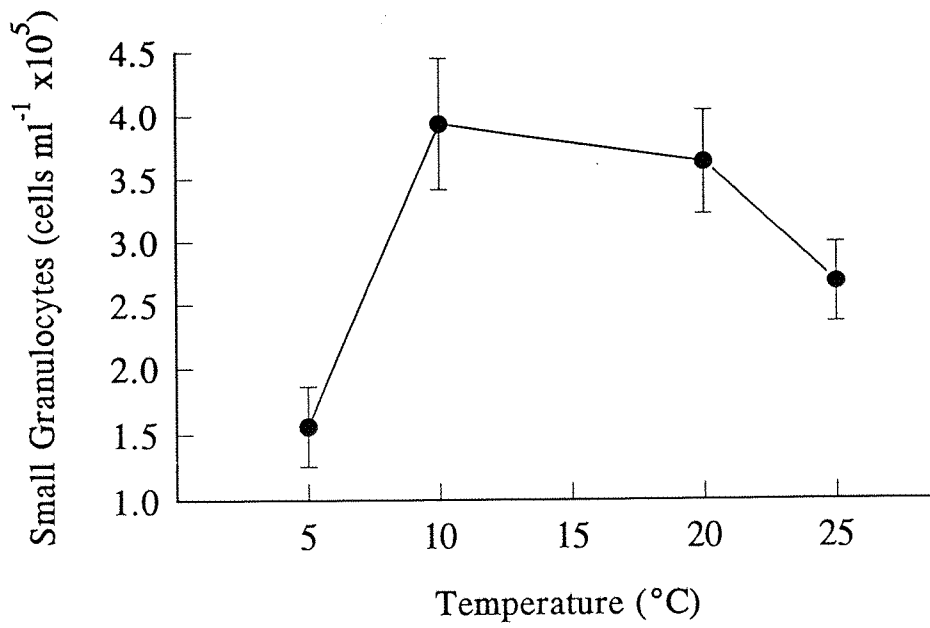


Figure 4.11 The effect of temperature on small granulocyte numbers (cells ml⁻¹ x 10⁵) of *Tapes philippinarum* at 33‰. Mean \pm SE, n = 12 at each point.

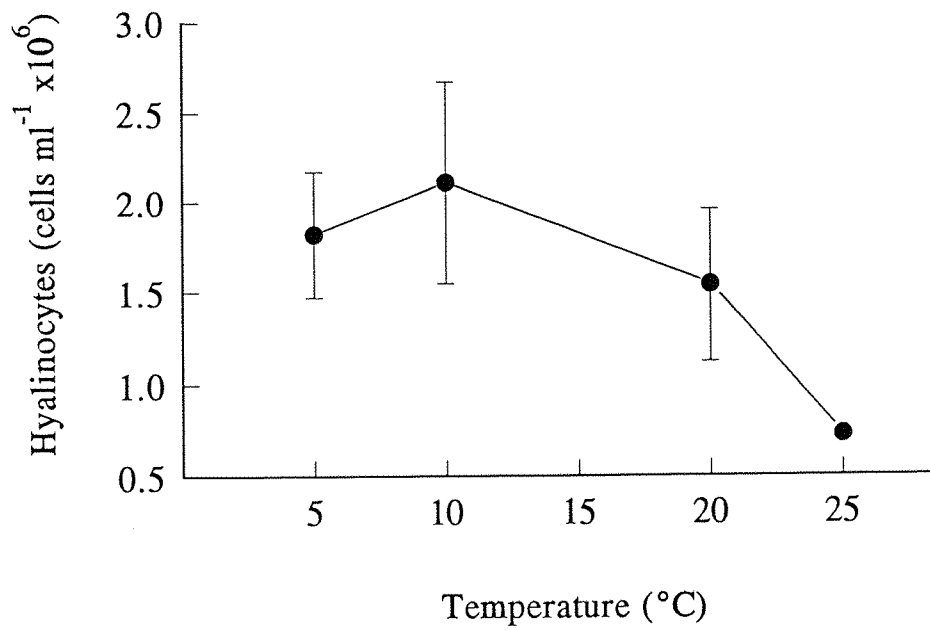


Figure 4.12 The effect of temperature on hyalinocyte numbers (cells ml⁻¹ x 10⁶) of *Tapes philippinarum* at 33‰. Mean \pm SE, n = 12 at each point.

temperatures tested ($P > 0.05$). The rate of locomotion of *T. philippinarum* amoebocytes is shown in figure 4.15. There was no statistically significant difference between any of the temperatures ($P > 0.05$). The values obtained for *T. philippinarum* were in the range 1.53 to 2.57 μm per minute.

For all temperatures between 5°C and 20°C the amoebocytes showed a decrease in cell size (figure 4.16). Only at 25°C was any increase in cell size observed with no statistically significant difference between any of the temperatures ($P > 0.05$).

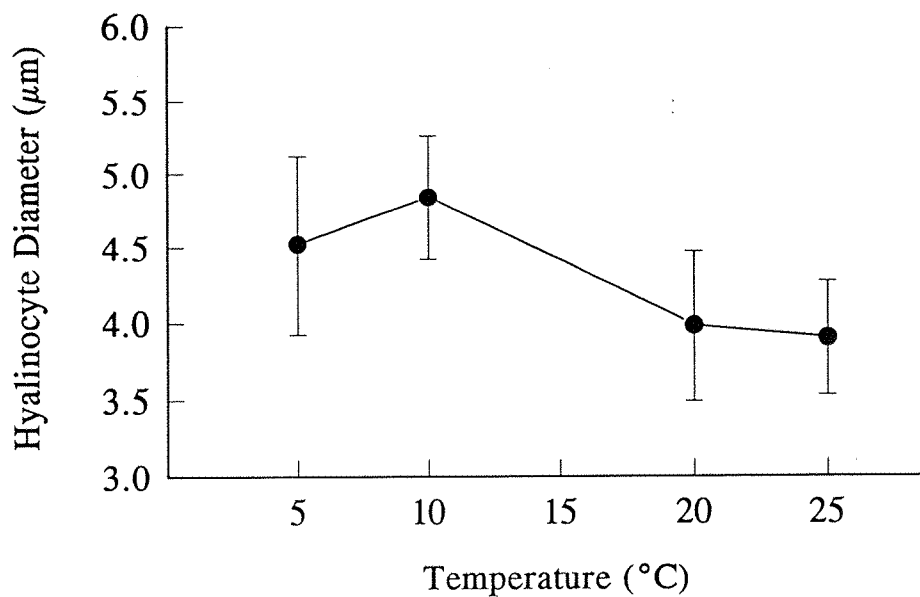


Figure 4.13 The effect of temperature on hyalinocyte diameter (μm) of *Tapes philippinarum* at 33‰. Mean \pm SE, $n = 12$ at each point.

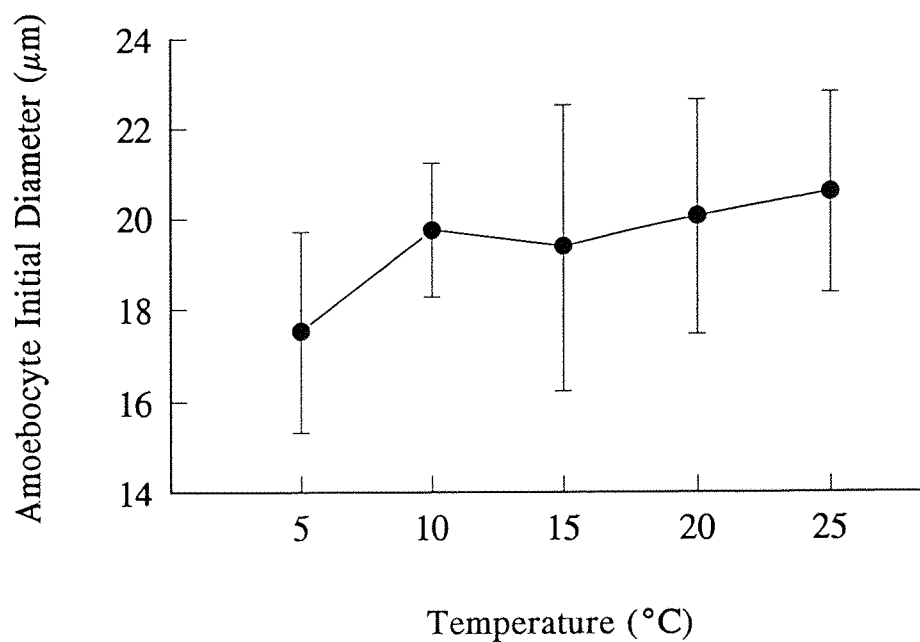


Figure 4.14 The effect of temperature on initial amoebocyte diameter (μm) of *Tapes philippinarum* at 33‰. Mean \pm SE, $n = 12$ at each point.

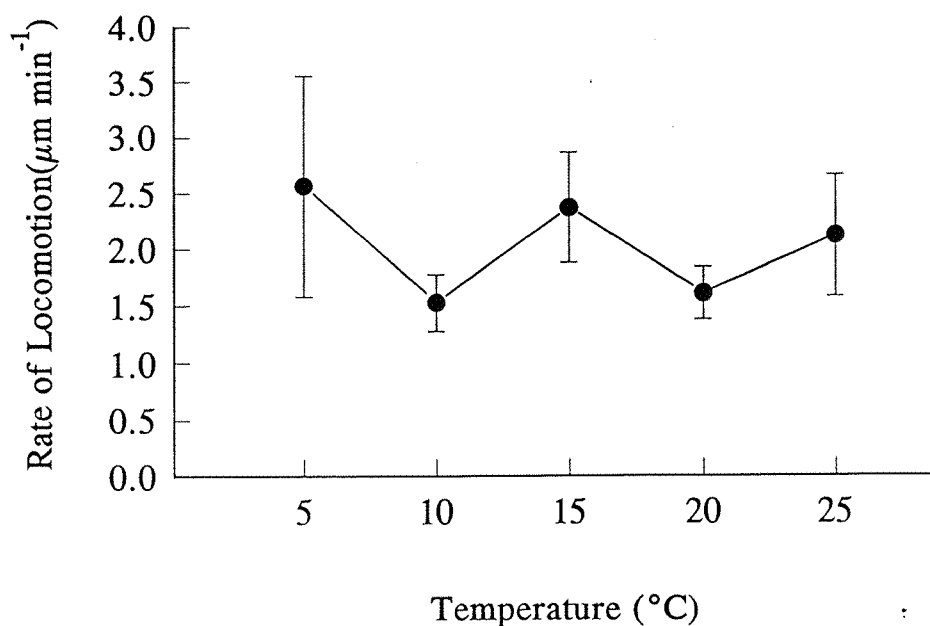


Figure 4.15 The effect of temperature on rate of amoebocyte locomotion ($\mu\text{m min}^{-1}$) of *Tapes philippinarum* at 33‰. Mean \pm SE, n = 12 at each point.

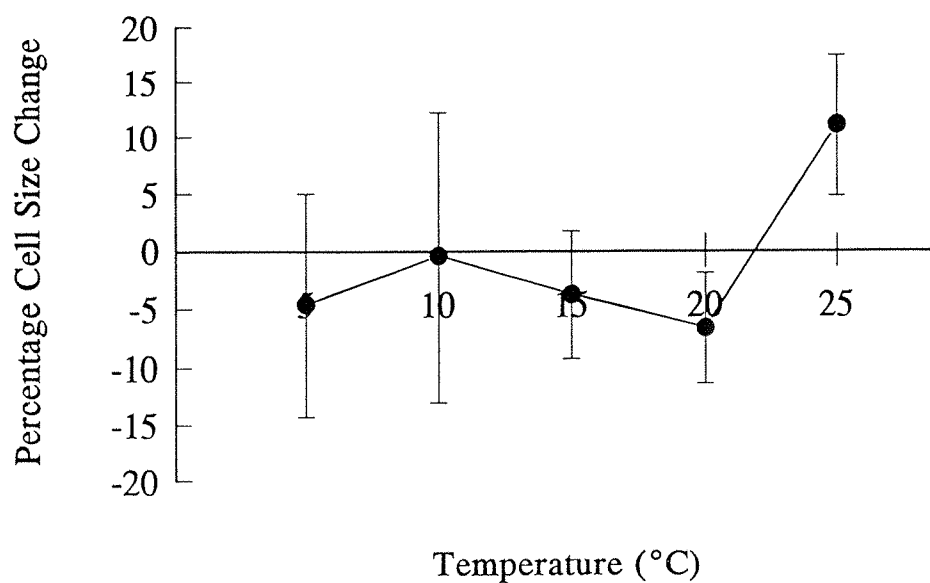


Figure 4.16 The effect of temperature on amoebocyte percentage cell size change of *Tapes philippinarum* at 33‰. Mean \pm SE, n = 12 at each point.

4.4 DISCUSSION

4.4.1 Scope for Growth in *Tapes philippinarum*

The use of the Manila clam *Tapes philippinarum* to broaden the range of study species not only provided valuable comparative data of an exotic species with the oysters studied but also provided important information on how this species copes in a range of environmental conditions. Although the study was limited to one salinity, chapter 2 showed how temperature tends to be the dominant influencing factor in physiological changes. The filtration rate (figure 4.01) showed a non-linear relationship with maximum rates occurring at 15°C and 20°C. This matched observations made by Goulletquer *et al.* (1989) who also found that quantities of seston in the range 0 to 30 mg per litre had no effect on the filtration rate. It is possible that seasonal physiologies exist, as have been described for *O. edulis* and *C. gigas*, but it would be necessary for further investigations to be made (preferably over the same matrix of variables as for *C. gigas*) before definite conclusions could be drawn.

The large change in filtration rates observed between 10°C and 15°C suggests that any discontinuity in physiology occurs at a similar temperature to *O. edulis* (Hutchinson and Hawkins, 1992). The clam exists naturally in more tropical water so any winter-summer physiology change is likely to occur at a temperature similar to *C. gigas*. However, the original imported broodstock came from the more temperate waters of Washington State, U.S.A. (Utting and Spencer, 1992). A lower temperature for change in physiology may have become selected for in these clams, but why this should manifest itself only in this species and not in the Pacific oysters is unclear.

There does appear to be a substantial increase in absorption efficiency (figure 4.02) between 10°C and 15°C but this proved to be statistically non-significant. The range of efficiencies observed (74-85%) is similar to the maximum assimilation efficiency observed by Goulletquer *et al.* (1989) of 78%. They also found that ingestion of food by *T. philippinarum* was regulated by the production of pseudofaeces. Where the food ration was increased or decreased to extreme levels the assimilation efficiency was reduced. For this study the algal ration was maintained at constant concentrations, below the threshold for

pseudofaeces production, and quality, so observed variations were only a result of responses to changing temperature. Low efficiency during the winter may have a significant effect when the particulate load is high, especially if a high proportion is inorganic material. The reduced efficiency together with increased pseudofaecal output results in weight loss as stored reserves are used up.

Respiration rate (figure 4.03), as for filtration rate, showed a non-linear relationship with temperature. Gouletquer *et al.* (1989) also observed this but found maximum rates occurred at 20°C. This study showed high rates at 15°C and 25°C, with a slight although non-significant decrease at 20°C. There was, however, a statistically significant difference between the lower temperatures and the higher temperatures, lending further support to the possibility of discrete winter and summer physiologies. Size and stage of development are probably important factors in determining these physiological rates, as was recorded with *C. gigas*. Juvenile clams show optimal production and metabolic processes at 20°C to 25°C (Bodoy *et al.*, 1986; Laing *et al.*, 1987), whereas this temperature range triggers gametogenesis in adults which interferes with animal metabolism (Mann and Glomb, 1978). Optimum conditions for tissue production in adults were found to be between 13°C and 21°C, with maximum production at 16°C (Bernard, 1983). Gouletquer *et al.* (1989) found that excretion of organic nitrogen varied throughout the year. The excretion rate for *T. philippinarum* (figure 4.04) showed no linear relationship with temperature, and gave maximum levels at 20°C; the range of excretion rates exhibited by the clam (13.77 to 70.62 $\mu\text{M N h}^{-1} \text{ g}^{-1}$ dry weight) was slightly lower but otherwise very similar to that in *C. gigas* under the same conditions (24.93 to 83.46 $\mu\text{M N h}^{-1} \text{ g}^{-1}$ dry weight), possibly reflecting the adaptation to similar latitudes originally occupied by, and the current temperate location of the two species. Comparison of the other metabolic processes showed a similar range for respiration rates but a higher filtration rate range in *C. gigas* - the oyster also demonstrated a more linear relationship with a change in seasonal physiology at mid-temperature.

When the scope for growth (SFG) index (figure 4.05) was compared with its physiological components it was found that as with the two oyster species in this study, filtration rate had the most significant effect on the index. Table 4.01

also shows that excretion rate exhibits a significant correlation with SFG, although this was not found with other studies. Maximum SFG was demonstrated at 15°C to 20°C, the same range as described by Bernard (1983). Individual energy budgets for *T. philippinarum* clams showed that production was more dependent on temperature than on the energetic value of the food (Gouilletquer *et al.*, 1989), and other studies have shown temperature to have a high influence on Manila clam survival. Animals which endure freezing temperatures when exposed at low tides frequently exhibit high mortality (Bower, 1992). Mortality was recorded as occurring because of cell dehydration as a critical amount of tissue water is lost to extra-cellular ice formation. However, some clams are able to adapt to and tolerate these conditions, surviving as a result of modified cell metabolism and calcium dependent mechanisms (Murphy and Pierce, 1975). This again demonstrates the phenotypic plasticity and ability to adapt physiologically to a range of environmental conditions, making this bivalve another desirable commercial species for exploitation.

4.4.2 Body Condition Index and Energy Partitioning in *Tapes philippinarum*

The body condition index (BCI) of the Manila clam showed a highly significant, negative correlation with the SFG index (figure 4.06). The non-linear nature of the previous measurements resulted in a significant decrease to minimum BCI at 15°C. Bivalves generally exhibit a seasonal cycle to the storage and utilisation of biochemical reserves, with strong influence from water temperature, quality and quantity of the food available, and from the state of gonad development (Laing, 1993). The effects these variables have on BCI was highlighted in section 2.4.2, with reproductive condition playing a significant role in how SFG energy is used. The increasing BCI as temperature rises from 15°C may be indicative of the ripening of gonads as temperature begins to influence gametogenesis. The falling SFG supports this as energy is directed towards the gonad development and not somatic or shell growth. Unfortunately, the state of clam gonads was not recorded so this index cannot be correlated with temperature.

The low temperatures also gave high levels of BCI; Walne (1970) observed in oysters that individuals reared in cooler water tended to have higher body condition.

Figure 4.07 shows the relationship between the total amount of energy ingested and the different fates of that energy. The steady increase in energy lost through respiration and excretion contrasted with the sudden increase in ingested and assimilated energy between 15°C and 20°C. This change resulted in more energy available for SFG at higher temperatures, although at 25°C SFG was reduced as assimilated energy decreased and expended energy continued to increase. No negative SFG was indicated so energy resources were being supplemented all the time and not being used up. Overall, temperature tended to cause a gradual increase in energy available for somatic growth and gametogenesis, as was the case with *C. gigas* under these conditions (figure 2.08c). Differences in the two species were observed with the largest amount of SFG in the oyster occurring at 10°C to 15°C and a very small level of energy available at 5°C. *T. philippinarum* was able to maintain a more even pattern of energy intake and output, implying that it is hardier under winter conditions than the oyster is. This suggests the clam could colonise a wider range of latitudes although summer temperatures would become limiting when the clam needed to reproduce. Further experiments measuring the physiology of *T. philippinarum* over a range of salinities could indicate more optimal conditions for the clam, as in *C. gigas* (figure 2.07).

Earlier sections (2.4.3, 3.4.3) have discussed the manner in which *C. gigas* and *O. edulis* populations partition their ingested energy - the energy partitioning for *Tapes philippinarum* under the same conditions of 15°C/33‰ is shown in figure 4.08. The energy lost through respiration was very similar to that in Conwy and Solent *O. edulis*, but statistical tests showed that all the comparisons were significantly different, indicating the variability of individual animals. The energy lost in ammonia excretion also proved to be highly variable, with *Tapes* differing significantly from Conwy and Loch Sween oysters but not Pacific oysters. Energy lost through faeces and pseudofaeces was significantly different from Scottish and Pacific oysters, but this was not entirely unexpected as *T. philippinarum* is known

to regulate ingestion through the production of pseudofaeces (Goulletquer *et al.*, 1989). The resulting energy left for somatic growth and gametogenesis showed the Manila clam only to be significantly different from Loch Sween oysters which, as has already been discussed (section 3.4.3), show very low levels of SFG as a result of size.

The overall pattern demonstrated by *T. philippinarum* was very similar to that of the Conwy population of *O. edulis* and also to *C. gigas*. All these animals, for these experiments, originally came from the MAFF laboratory at Conwy and were collected from the same environmental physico-chemical regime. It may well be no coincidence that these animals exhibit similar partitioning patterns, and though the absolute energy requirements of each species are different and size differences are difficult to extrapolate between the species, the evidence appears to support the major influence the environment and its associated variables have on SFG.

4.4.3 Haemocytic Indices in *Tapes philippinarum*

The application of haemolymph assays is useful because parasitic disease often infects haemocytes in the haemolymph during the early stage of its infective cycle, long before other tissues become infected. The MSX parasite *Haplosporidium nelsoni* is detected in 90-98% of cases through haemolymph analysis (Gauthier and Fisher, 1990). Haemocytes spread the disease around the body and heavy infection can lead to the occlusion of blood sinuses and atrophy of vital organs. This type of assay allows the quantification of infection in a known volume, the early detection of infection, and the diagnosis of disease at systemic levels rather than localised foci. The use of anaesthetics such as magnesium chloride can also mean that animals need not be sacrificed.

In this study, where physiological measurements have been quantified, the use of additional indices has added to the suite of methods employed and provides valuable data for use in predicting harmful or stressful conditions. The total haemocyte counts for *T. philippinarum* are presented in figure 4.09. The large error bars indicated the high degree of variability in the samples, and it was

probably this which resulted in the lack of statistically significant differences between the groups. The range of haemocyte numbers (1.20×10^6 to 2.87×10^6 cells per ml) was similar to the count of 1.15×10^6 cells per ml found for *Mercenaria mercenaria* at $15^\circ\text{C}/33\text{‰}$ (Brooks, 1989). Unfortunately, data were missing for 15°C resulting in the peak in cell numbers occurring at 10°C . *C. gigas* showed no significant variations in total cell numbers with respect to temperature (Hawkins *et al.*, 1993b) and had very similar counts at 10°C and 15°C . The large granulocyte (amoebocyte) numbers showed no significant differences between any groups (figure 4.10), probably owing to the large variations recorded. A lack of variation was also observed in *C. gigas* amoebocytes (Hawkins *et al.*, 1993b) and it was this lack of variation which was attributed as the cause of stability in total haemocyte numbers.

The small granulocyte numbers shown in figure 4.11 showed a dramatic, significant increase from 5°C to 10°C & 15°C and again the maximum numbers of cells occurred at 10°C . This may have been an artifact of separate winter and summer physiologies; in winter all metabolic processes are slowed down, including haemopoiesis and this could account for low cell numbers observed at 5°C . Above 10°C , in the summer physiological state, the rate of turnover of haemocytes would be much greater. Replacement cells would be readily available for coping with pathogen challenge, possibly resulting in less cells in circulation. At high temperatures the majority of energy would be directed into gametogenesis, rather than maintaining the immunocompetence of the animals which is a somatic process resulting in reduced cell numbers. If data were available for 15°C it would be expected that this would show the highest cell numbers as this coincides with maximum SFG energy available for somatic production. The hyalinocytes (figure 4.12) matched the patterns observed for other cell types with maximum levels at 10°C and then a general decrease with temperature. Hawkins *et al.* (1993b) demonstrated a slight upward trend in all haemocyte counts for *Crassostrea gigas* although both these species have shown very little significant variation between groups. The study of the Pacific oyster demonstrated that salinity had far more effect on immunocompetence.

The lack of significant differences between temperatures for hyalinocyte

diameter (figure 4.13) was not unexpected as *C. gigas* also showed little change in diameter with temperature (Hawkins *et al.*, 1993b). Temperature, therefore, had no significant effect on the size of cells responsible for the production of hydrogen peroxide, so any variation in this immunological defence mechanism must have been the result of other influencing factors, or temperature affecting a different part of the chemical process of peroxide production. Temperature also had no significant effect on the initial diameter of amoebocytes, before their rates of locomotion and size change were measured (figure 4.14). This measure is part of an index of the phagocytic capacity and illustrated how temperature affects the engulfing and encapsulation of foreign bodies. *C. gigas* again showed little effect by temperature on initial diameter and was only influenced by low salinity (Hawkins *et al.*, 1993b). The use of diameter rather than areal measurements produced less variability, but the error bars in figure 4.14 showed how individuals could still differ considerably. Rates of locomotion (figure 4.15) also lacked significant differences, a trait mirrored by observations in *C. gigas*, although *O. edulis* was shown to be more sensitive to changes in conditions (Hawkins and Hutchinson, 1990). The range of locomotion rates observed in *T. philippinarum* (1.53 to 2.57 μm per minute) was considerably lower than the 6.6 μm per minute observed in *M. mercenaria* at 15°C/33‰ (Brooks, 1989). There was also a tendency for the amoebocytes to shrink with time at all temperatures below 25°C (figure 4.16). This suggests the cells were not in very good condition but the differences between all groups were non-significant. No decrease in cell dimensions was observed in *O. edulis* or *C. gigas* (Hawkins *et al.*, 1993b).

The observations made for all these haemocytic measurements showed that temperature has very little significant effect at high salinity. The use of a matrix of temperature and salinity measurements would provide a better data set and indicate whether these conditions are representative of high or low immunocompetence. It may be that the results here show a difference between winter and summer temperatures, possibly owing to different physiologies, but definite conclusions cannot be drawn from the limited range of data. When these measurements are considered in conjunction with the physiological observations, it is possible to surmise that high temperatures result in gametogenesis, reducing SFG and cell

numbers and thus making the animal more susceptible to disease. Low temperatures allow basic metabolism and haemocyte functions to be maintained, resulting in an optimum range of temperatures for *Tapes philippinarum* of 10°C to 20°C at 33‰.

4.4.4 Summary

The results obtained for the investigation of *Tapes philippinarum* were very similar to those obtained in the study by Goulletquer *et al.* (1989). All the physiological parameters showed non-linear relationships with temperature, and as in the measurements of the Ostreidae, it was filtration rate which had the most significant effect on scope for growth. It is possible that this species exhibits discrete winter and summer physiologies, as *Crassostrea gigas* and *Ostrea edulis* do. However, the use of a data set of five temperatures and a single salinity does not provide sufficient evidence for this. The rates measured showed similar values to those measured in populations of *C. gigas* and *O. edulis* taken from Conwy, although the oysters demonstrated a greater range of filtration rates. The body condition index of *T. philippinarum* was inversely proportional to the scope for growth index, and as with the other measurements was non-linear with respect to temperature. The increasing body condition index from 15°C occurred as a result of ripening gonads with temperature influencing gametogenesis.

Tapes philippinarum demonstrated an energetic balance across the temperature range similar to the found in *Crassostrea gigas*. The clam maintained a steady balance of energy uptake and output at low temperature, compared with the oyster, possibly demonstrating this species to have better adaptation to these conditions, making it a hardier winter animal. The energy partitioning of *T. philippinarum* is very similar to the other species from Conwy under the same environmental conditions. This gives further evidence to suggest that it is environmental parameters which have the most significant effect on scope for growth.

The haemocytic indices showed large variability, although the figures obtained were similar to previous studies of clams. The numbers of small

granulocytes showed possible summer/winter effects, possibly as a result of a change in haemopoietic rate. Temperature had little effect on the immunology, but it has been shown in *C. gigas* that salinity is the major influencing factor in haemocytic indices, further demonstrating the need to measure these parameters over a matrix of environmental variables.

CHAPTER FIVE

POPULATION GENETICS IN THE OSTREIDAE

CHAPTER 5

POPULATION GENETICS IN THE OSTREIDAE

5.1 INTRODUCTION

5.1.1 The use of Genetic Analysis in the Marine Environment

The role of genetics, and especially the development of electrophoretic techniques, has greatly increased our knowledge of population structures throughout the plant and animal kingdoms. The use of these techniques has demonstrated genetic variation within and between populations and when coupled with other methods of analysis, such as physiological parameters, has enabled the development of further hypotheses about genotypic-phenotypic relationships. Moreover, the science of genetics encompasses such traditional disciplines as biochemistry, molecular biology, cytology, physiology and ecology, and how these fields influence the inheritance of genetic information by progeny. Knowledge of the way these processes interact is often used as a tool in the selective breeding mechanism, but when they are applied individually, they may not produce the genetic gains required. Mallet *et al.* (1986) indicated that, "*understanding the role of genotypic variation in relation to diverse production patterns characteristic of natural populations is an important issue for marine systems. Natural selection acts on the phenotypic variation to produce either characters with low variability (homeostasis) or characters with high variability (plasticity)*". This indicates that short term responses to variable environmental conditions will be observed as changes within the components of production, for example in shellfish as meat yield, shell length and weight, and percentage metamorphosis and survival.

Koehn (1984) identified three of the major problems in applying genetic principles to the marine environment as,

- 1) an identification of the biotic and physical factors which determine the abundance of marine organisms,
- 2) an estimation of the effects of man's activities on the biology of marine

species,
and 3) a determination of the strategies to be employed in the rational exploitation of the marine environment.

It is a basic assumption when using enzyme electrophoretic methods that the genetic diversity of enzymes represents phenotypic diversity, and that natural selection can potentially discriminate between alternative phenotypes and thereby indicate genetic differences within and between populations. Biochemical diversity is also presumed to have a significant effect upon higher levels of biological organisation resulting in specific genotypic fitnesses. Enzymes have identifiable functions that are easily quantifiable under different environmental and physical conditions, and consequently, research over the years has been directed towards examining allozyme variation as a mechanism of population differentiation. However, in the last ten years studies have moved on to examine the genetic components of life history and demographic variation, such as fecundity, fertility and longevity; these are reviewed later in this chapter.

This genetic study addresses some of the recommendations made by Koehn (1984) for the application of genetics to problems in the marine environment, and in particular the statement that,

"In general, a better understanding is required of environmental influences on gene function and metabolism. Attempts should be made to more fully integrate genetics with traditional physiological ecology. The genetic basis of physiological variation and the physiological consequences of genetic variation are virtually unknown. Studies of the physiological responses to stress should be combined with genetic information to elucidate how different genotypes influence adaptation to stress".

In chapter 3 populations of *Ostrea edulis* were shown to have different physiological responses to the same environmental conditions, possibly indicating discrete ecotypes. The underlying explanations for this may be influenced solely by the 'normal' environmental regime, and the acclimation of the oysters to these conditions, or it may have a genetic basis so successive generations have a genetic make-up more suited to particular conditions (or more likely, there is a combination of influencing factors, i.e. selective pressure). In this study, gel-

electrophoresis was employed to estimate,

- 1) the level of genetic variation in different geographical populations of *O. edulis*,
- 2) inter-specific genetic variation in oyster species,
- and 3) the genetic identity of the *O. edulis* populations (i.e. what similarities and/or differences are present).

With these measurements that quantify both genotype and phenotype, it is possible to erect an hypothesis as to the influence of genetic factors on phenotypic expression. Initially it was assumed that the Solent and Conwy populations would be most similar, since the English oysters were used as broodstock for Conwy (see sections 3.1.2 and 3.1.3). The Scottish oysters have had a much more varied mix of stocks, so it was presumed that these oysters would show more genetic variation than the other populations.

5.1.2 Previous Studies

In recent years electrophoretic methods have been extensively employed to examine a variety of molluscan traits. In addition to profiles of genetic variation, differentiation and similarity (e.g. Buroker *et al.*, 1979a; 1979b; Ward and Warwick 1980; Buroker, 1982; Zouros and Foltz, 1984; Johannesson *et al.*, 1989; Beaumont and Khamden, 1991; Saavedra *et al.*, 1993) allozyme variation has also been used for systematic analysis (Buroker *et al.*, 1983), growth rate (Zouros *et al.*, 1980; Koehn and Gaffney, 1984; Diehl and Koehn, 1985), evolutionary patterns (Buroker, 1985) and other studies besides. Those studies focusing specifically on *O. edulis* are listed in Saavedra *et al.* (1993).

The gradual process of isolating populations during evolutionary divergence has been divided into 5 stages by Ayala *et al.* (1975). These are, in order, the formation of:

- i) geographical populations
- ii) sub-species
- iii) semi-species
- iv) sibling species

v) non-sibling species.

According to these criteria, the three populations of *O. edulis* used in this study can be considered to fall into the first category. It has been shown for other studies of oysters that different geographical populations exhibit high degrees of genetic similarity (Buroker *et al.*, 1979a; 1979b; Johannesson *et al.*, 1989). Buroker *et al.* (1979a) used Nei's index of similarity (Nei, 1972) to show that populations of *Crassostrea gigas* in the Sea of Japan and separated by 750 km, were genetically very similar, as was the case in *Saccostrea commercialis* populations of Australia and New Zealand, separated by 1200 km. Buroker *et al.* (1983) also showed that oysters with the same genotype can appear phenotypically different. Different environmental conditions give rise to different ecomorphs¹ of the New Zealand dredge oyster *Tiostrea lutaria*. When the genetic identity of this population was compared with another species (*T. chilensis*), with a different geographical location, both species were found to be genetically very similar. The low genetic variation and changeable morphology demonstrate the phenotypic plasticity of oysters, rather than selection for particular traits.

The life history of *O. edulis* also determines the degree of genetic variation between and within populations. Buroker (1985) showed that larviparous oysters, i.e. those which brood larvae before releasing veligers into the water column, tend to inhabit a narrower geographical range, leading to increased inbreeding and lower genetic variability. This strategy could lead to the elimination of populations in severe conditions, for example with the spread of disease. Conversely, oviparous species (those which release eggs directly into the water column) tend to have a longer planktonic period allowing dispersal over a wide area, giving rise to enhanced gene flow and therefore a greater degree of genetic variation. There is also less likelihood of a population being wiped out by a local catastrophe such as disease outbreak. By using heterozygosity as a measure of genetic variation

¹It should be noted that the term 'ecomorph' is used to describe animals which differ in their morphology, i.e. shell shape, as a result of changing environmental conditions. The term 'ecotype', used later in this chapter, refers to animals which are different not only in their morphology, but also in their physiology, metabolism and/or their immunology as a result of different environmental conditions.

Buroker (1985) was able to demonstrate a significant correlation between variation and heterozygosity, and a non-significant correlation with body size. This was also reported in the study by Zouros *et al.* (1980), in which individual weights were correlated with the number of loci at which the individual was heterozygous, and it was found that the mean weight of heterozygotes was larger than the mean weight of homozygotes.

The most recent genetics survey of *O. edulis* populations was by Saavedra *et al.* (1993) who examined eleven different sites around Europe. They confirmed the low levels of genetic variation between the populations, attributing this to the brooding habit of the animal (*vide* Buroker, 1982). The long term effects of this are to reduce population size as well as the level of genetic variation.

However none of the aforementioned studies have looked at populations from Britain. The restriction on movement of oysters between sites (the Molluscan Shellfish Control of Deposit Order 1974) because of bonamiasis and other possible disease transfer, has effectively isolated certain growing areas. In the present study, genetic comparisons were made between populations from three of these areas - from Loch Sween in Scotland, Conwy, North Wales, and from the natural population in the Solent.

The measurement of scope for growth (SFG) in these populations (chapter 3) may be considered as a method of quantifying an aspect of phenotypic 'fitness'. The use of enzyme electrophoresis would provide complimentary information on the genetic variation within British populations, and the degree to which variation in SFG may be attributed to genotypic differences or phenotypic plasticity.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Samples

A variety of extraction/homogenisation techniques were tested to determine which was the best method for giving clearest separation upon electrophoresis. After testing individual tissues it was decided to use the whole animal for each assay as there were no differences in isozyme patterns for whole tissue compared with individual tissues, and the whole animal gave sufficient material for analysis.

The animal was removed from its shell, and excess moisture was blotted off with paper. This soft tissue was carefully wrapped in cling film™ and immediately frozen in liquid nitrogen, to stop any further metabolic processes from occurring and altering the states of the enzymes being studied. The frozen oyster was then ground up with a steel mortar and pestle, pre-cooled in the liquid nitrogen, and the resulting fine powder re-suspended in 5-10 ml of distilled water, before thorough homogenisation with an Ultraturax™ homogeniser. This homogenate was spun down in a refrigerated centrifuge, for 5 minutes at 1800 g, and the supernatant pipetted into small vials and frozen at -20°C. Freezing was found to improve the freeze drying stage and gave better resolution on electrophoresis. Samples were lyophilised overnight in a Lyoprep™ 3000 freeze dryer, after which they were stored at -20°C for later use. This protocol is summarised in figure 5.01. The enzymes chosen to be examined by electrophoresis were based on those used in previous studies and which were found in preliminary experiments to give good resolution on staining. These enzymes are listed in table 5.01. The enzymes were analysed using methods modified from Harris and Hopkinson (1976), on polyacrylamide gels.

5.2.2 Poly-Acrylamide Gel Electrophoresis (PAGE)

The formulation for polyacrylamide gels was based on the method given in Harris and Hopkinson (1976). For each run 50 ml of gel solution was made which was enough for two gels of 10 samples each; compositions of the various gels used are given in Appendix A2. This solution mixture was poured into a 50 ml syringe and then poured carefully (so no air bubbles were formed) into the gel mould.

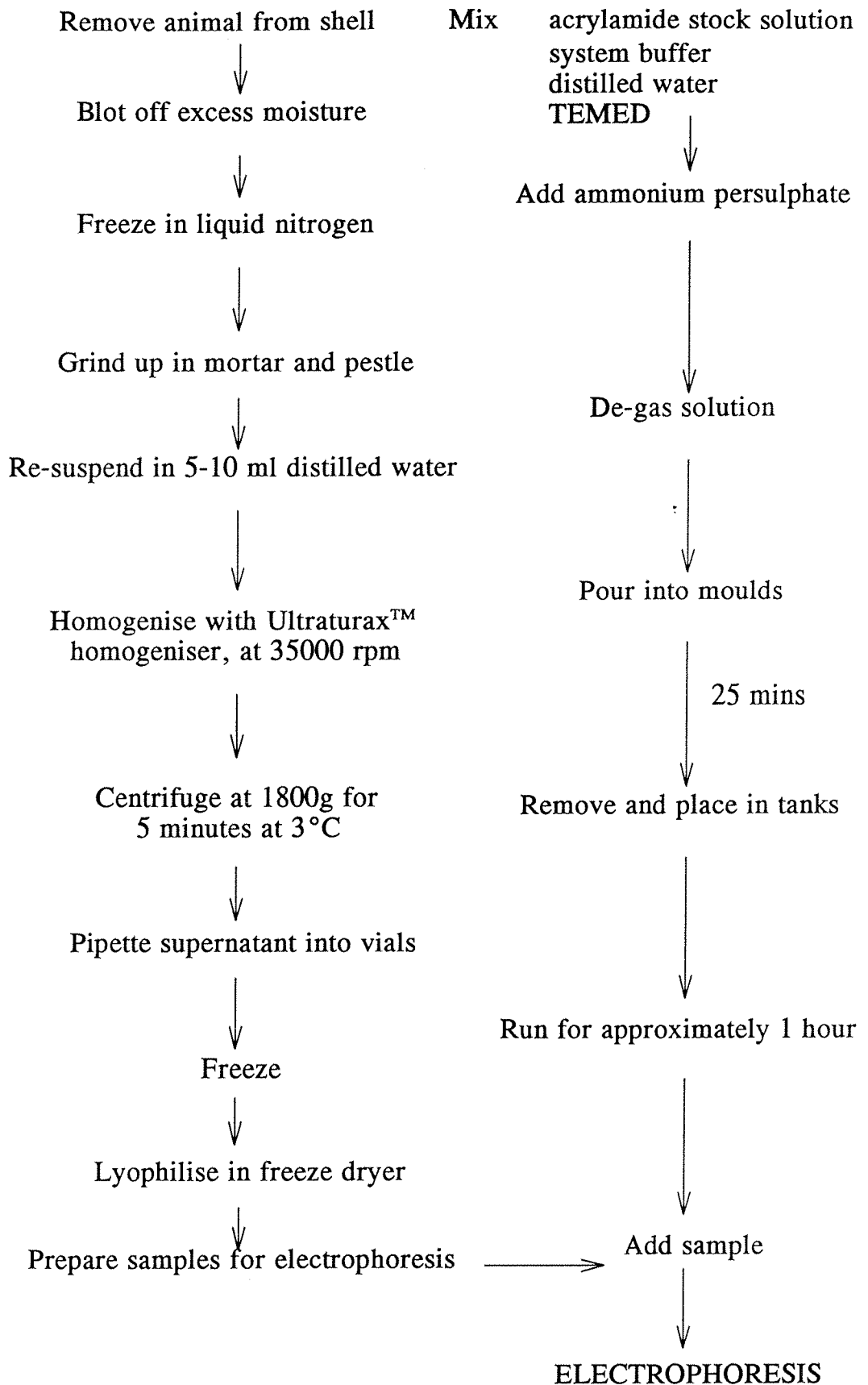


Figure 5.01 Protocol for the preparation of tissue samples and polyacrylamide gels for electrophoresis.

	Enzyme	Abbreviation	Enzyme Number	Buffer System	Location of enzyme in metabolic pathway
i)	Lactate Dehydrogenase	LDH	E.C. 1.1.1.27	1	Glycolysis
ii)	Malate Dehydrogenase	MDH	E.C. 1.1.1.37	2	TCA balance
iii)	Isocitrate Dehydrogenase	ICD	E.C. 1.1.1.42	2	Redox balance
iv)	NADH Diaphorase	DIA	E.C. 1.6.2.2	3	Redox balance
v)	Superoxide Dismutase	SOD	E.C. 1.15.1.1	1	Detoxification
vi)	Adenylate Kinase	AK	E.C. 2.7.4.3	3	Adenylate Catabolism
vii)	Phosphoglucomutase	PGM	E.C. 2.7.5.1	4	Pre-glycolytic
viii)	Esterase	EST	E.C. 3.1.1.1	4	Fatty Acid Metabolism
ix)	Leucine Aminopeptidase	LAP	E.C. 3.4.11	5	Protein Catabolism
x)	Phosphoglucose Isomerase	GPI	E.C. 5.3.1.9	3	Glycolysis

Table 5.01

Enzymes used in electrophoresis. The buffer systems used were : 1) Bridge 0.2 M phosphate, pH 7.0, Gel 1:10 dilution of bridge;
2) Bridge 0.245 M NaH_2PO_4 / 0.15 M Citrate, pH 5.9, Gel 1:40 dilution of bridge; 3) Bridge 0.1 M Tris / Citrate, pH 8.0, Gel 1:5 dilution of bridge; 4) Bridge 0.1 M Tris / 0.1 M Maleate / 0.01 M Na_2EDTA / 0.01 MgCl_2 , pH 7.4, Gel 1:10 dilution of bridge;
5) Bridge 0.1 M Tris / 0.1 NaH_2PO_4 , pH 7.4, Gel 1:20 dilution of bridge.

Gels were found to be set firm after 5-10 minutes. The concentration and type of gel buffer is listed in table 5.01. A current was passed through the gel for about 30 minutes before any samples were applied to ensure there was no persulphate inhibition of allozyme separation.

Running conditions were chosen using the lowest voltage and longest run time for each enzyme separation as detailed in Harris and Hopkinson (1976). The mean run time for these separations was 16 hours, with the voltage gradient in the range 7-10 V cm⁻¹. All the runs were made using cooling plates at approximately 3°C ± 1°C. Bromothymol blue solution (0.04 % w/v) was used as a front marker. Extracts were prepared for electrophoresis by dissolving the lyophilised samples in 100 µl of bridge buffer. 5 µl aliquots were applied to each sample well. Sodium dodecylsulphate (SDS) electrophoresis was also tested to identify structural proteins but was found to be insufficiently sensitive to give quantifiable results.

5.2.3 Staining Procedures

The composition of stains and staining methods for each enzyme were taken from Harris and Hopkinson (1976). It was important that the gels were left in the staining solution to allow any bands to develop properly. Where possible the stain was applied as a 2% (w/v) agar overlay rather than as a fluid stain, to reduce and eliminate patchiness in the staining. Where enzymes had alternative methods for staining, the following modifications were used :

AK	- was stained with MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and PMS (Phenazine Methosulphate).
PGM	- was stained with twice the quantity of glucose 1-phosphate and glucose 6-phosphate dehydrogenase, to detect the fainter PGM-3 isozyme.
EST	- was stained using the α naphthyl acetate and Fast Blue B system.
LAP	- was stained using an L-amino acid in the presence of snake

venom L-amino acid oxidase, peroxidase and 3-amino-9 ethylcarbazole.

5.2.4 Analysis of the Gels

When the bands had developed they were photographed immediately with a video camera to give a video tape record. These video images were digitised for image analysis using the JAVA™ system (JANDEL software). A pseudo-colour scale was applied to the image to emphasise differences in banding intensities, and the image was 'smoothed' to provide a clearer picture. A vertical average of band optical density (in arbitrary density units) was taken across the width of a well (equal to the width of a band) from a point behind the well to the front of the migrated bands. This was repeated for each sample on the gel (between the same start and finish points) to give data for each sample run. When plotted, the data provided a series of peaks of intensity where isozyme bands were located. Plots were produced for :

- 1) y-scale (intensity) with an optimum range, to show bands for each individual sample (i.e. banding patterns),
- and 2) y-scale (intensity) with the same range for all samples (on one gel) to allow comparisons to be made between the intensities of each sample (i.e. comparative densities).

An example of the type of plot obtained is presented in figure 5.02. Each locus (e.g. DIA, PGM etc) had two gels for each run, so were numbered #1 and #2. (These numbers do not identify different loci for a particular enzyme.) For each sample the number of alleles (bands) was identified by eye and marked. The corresponding position of each band in each sample was used to equate bands between samples. The frequency of each band within populations or species of an animal was determined for all available gel samples (eg DIA #1 and DIA #2), and these were averaged to give the frequency of each allele for each locus. Rf values were determined by measuring the distance between the middle of the well and the middle of the front of the appropriate band. The migration front in each plot was taken as the limit of the x-axis, with means taken for Rf values for each population

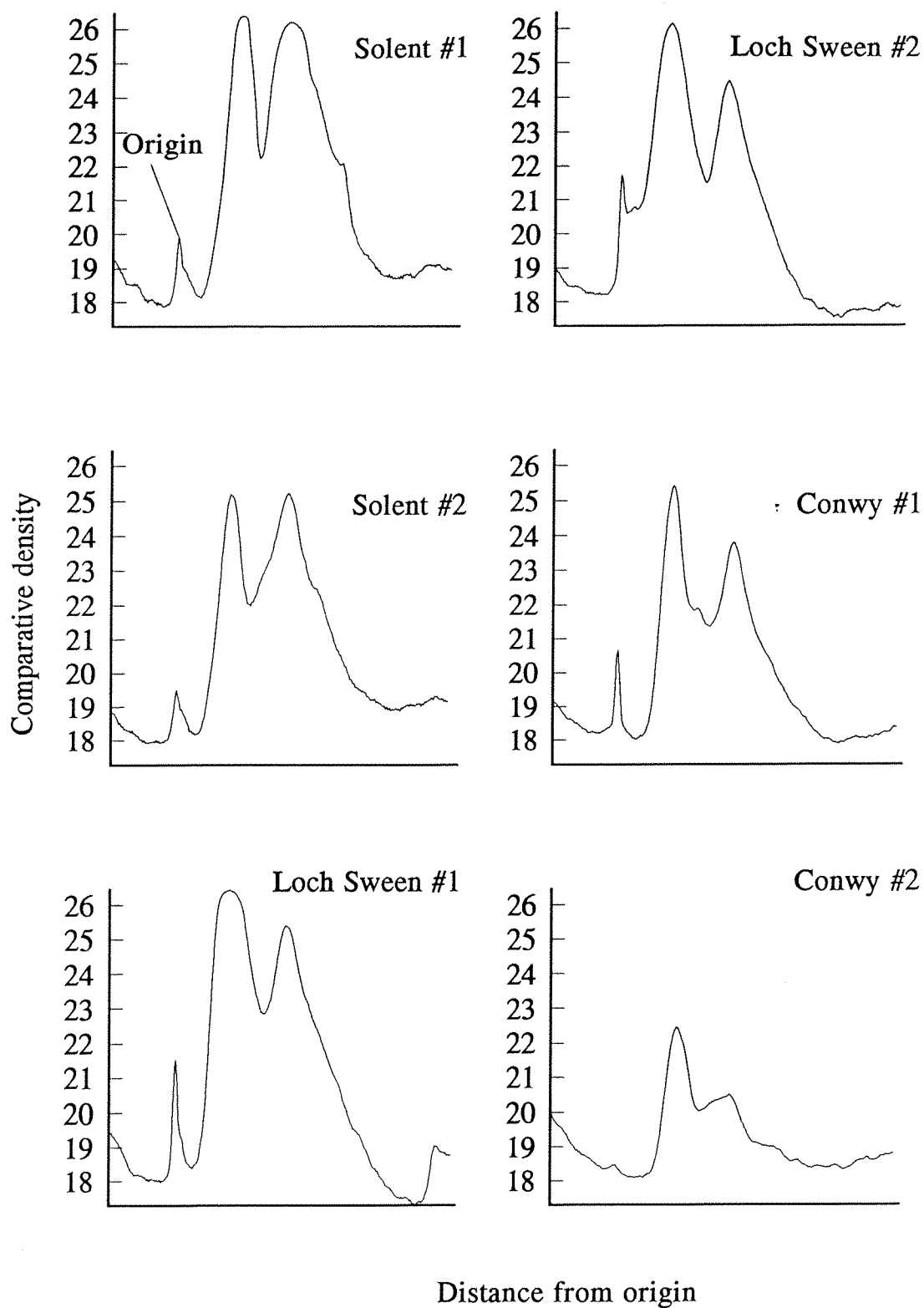


Figure 5.02 Example of micro-densitometric analysis of enzyme electrophoresis (malate dehydrogenase).

or species for each enzyme.

5.2.5 Genetic Indices and Data Analysis

Mean numbers of alleles per locus were calculated for each population, which together with other indices, gives a measure of the genetic variation within the populations. The mean frequencies of each allele were used to calculate :

a) DEPARTURE FROM EVENNESS OF ALLELE FREQUENCIES (E)

The estimation of evenness of allele frequency distribution (E), as defined by Johnson and Feldman (1973), gives a measure of non-neutrality, i.e. alleles which after mutation may have a positive or negative selective effect. The value of E depends upon the number of alleles and it has been shown that the only loci which exhibit large numbers of alleles are those whose enzymes utilise substrates originating directly from the environment (Kojima *et al.*, 1970; Johnson, 1972, as cited in Johnson and Feldman, 1973). Consequently this may be used as a measure of the effect which the environment has on the loci being studied.

$$E = K \sum x_i^2$$

where K = actual number of alleles observed

and x_i = frequency of the i th allele

b) GENETIC IDENTITY (I)

The degree of similarity between sets of allozyme data from two populations was estimated from the normalised probability of the identity of alleles (Nei, 1972). When the genetic similarity between two sampling groups is 1.0, the populations are genetically identical. It can be used to calculate the genetic distance

between two populations.

$$\text{Similarity} \quad I = \frac{\sum x_i y_i}{\sum x_i^2 y_i^2}$$

where x_i and y_i represent the frequencies of the i th allele in the x and y populations respectively.

$$\text{Distance} \quad D = -\log_e I$$

c) POLYMORPHISM VALUE

This is the number of polymorphic loci divided by the total number of loci within the population (Buroker, 1985), and gives an estimate of the number of loci which exhibit genic variation from a random sample of the genes in a population. Thus the amount of polymorphism is an estimate of the genetic variation at the population level.

5.2.6 Statistical Analysis of Results

The results of laboratory experiments were expressed as mean values \pm standard deviation, unless otherwise stated. The number of observations at each datum point was in the range 6-30. Where appropriate data were analysed using an unpaired t-test (or a Mann-Whitney test as a non-parametric alternative) for comparing two groups, or a standard ANOVA (or a Kruskal-Wallis test as a non-parametric alternative) for comparing more than two groups.

5.3 RESULTS

From the electrophoresis performed, activity was only found at seven of the ten designated loci for the study (MDH, SOD, EST, PGM, GPI, DIA, and ICD). The time constraints of the study curtailed analysis of the three undetected loci. Each band detected within the gels was taken to represent a single allele. The frequencies of these alleles was calculated and used to estimate the mean alleles per locus, represented in table 5.02. Statistical analysis showed the differences to be non-significant ($P > 0.1$). These data were used to calculate genetic variation within the populations, expressed as polymorphic loci per population (table 5.02).

	Solent	Loch Sween	Conwy	<i>C. gigas</i>
No. of loci studied	10	9	9	2
No. of loci with detectable activity	7	6	6	2
Mean alleles per locus	2.174 ± 1.604	3.667 ± 1.033	3.000 ± 1.581	2.500 ± 0.707
Polymorphic loci per population	0.267	0.263	0.105	?

Table 5.02 Amount of allelic activity and estimates of polymorphism for three populations of *Ostrea edulis* and of *Crassostrea gigas*.

Pairwise comparisons for all the loci were made amongst the three populations and a mean genetic identity was found. These similarities, and the proportion of loci sharing them are shown in figure 5.03. Using Nei's index of similarity, a genetic identity of 0.929 ± 0.208 was found for the three

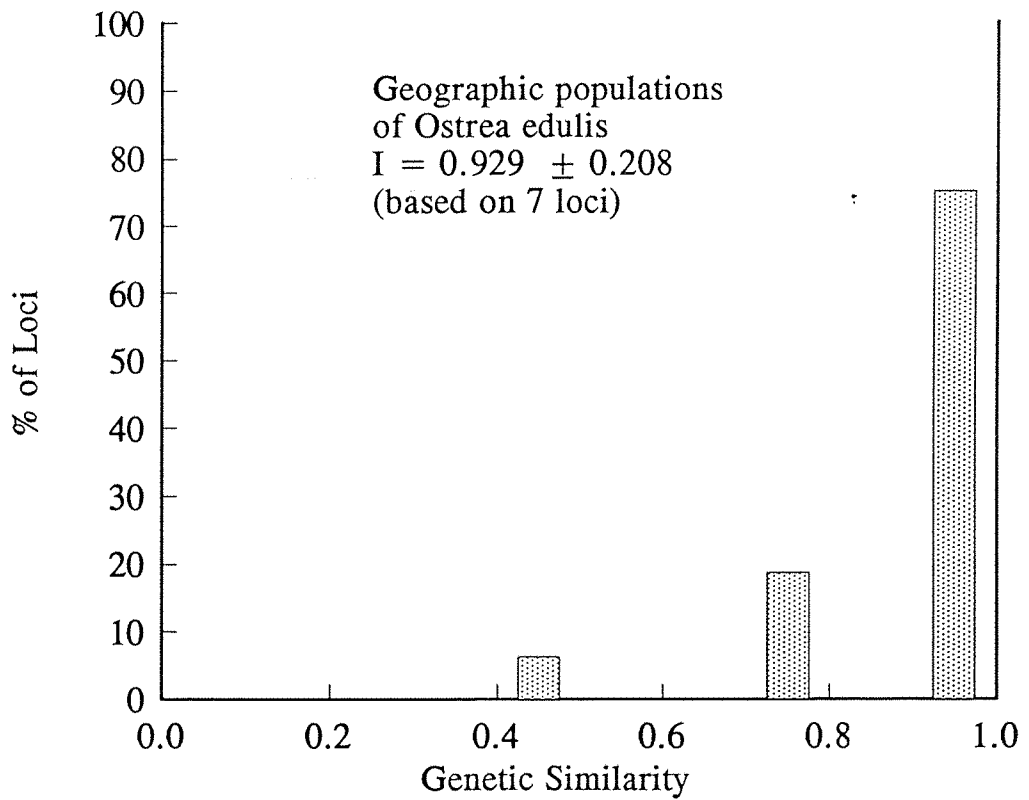


Figure 5.03 Distribution of loci with respect to genetic similarity. Comparisons of homologous loci were made between the populations from Loch Sween, Conwy and the Solent. I = genetic identity \pm standard deviation.

populations. When used to calculate genetic distance, a measure of the accumulated number of codon differences per locus, a value of 0.0999 ± 0.249 was obtained, i.e. on average, less than 0.1 electrophoretically detectable allelic substitutions per locus occurred between these different geographical populations of *O. edulis*.

Nei's indices of similarity and distance were calculated for the three populations of *O. edulis* at each locus (table 5.03) and an overall index for similarity and distance based on these values is represented in table 5.04.

	Genetic Identity (I)			
	Solent vs Loch Sween	Solent vs Conwy	Loch Sween vs Conwy	Solent vs <i>C. gigas</i>
MDH	0.913	0.987	0.953	-
ICD	1.413	0.969	0.984	-
DIA	0.950	0.788	1.155	-
SOD	0.707	1.000	0.707	-
PGM	0.980	0.968	0.942	-
EST	-	-	-	0.987
GPI	0.452	-	-	0.507

Table 5.03 Estimates of genetic similarity (I) for three populations of *Ostrea edulis* and for *Crassostrea gigas*, measured at individual loci.

Table 5.05 represents a further index of genetic variability with Johnson and Feldman's departure of evenness from allele frequencies. The values of E, with the exception of SOD, were all greater than one - the larger the value of E, the greater the skew of the observed frequencies away from evenness. T-test and one way ANOVA again showed there were no significant differences ($P > 0.05$) between populations.

	Solent	Loch Sween	Conwy
Solent		0.903 ± 0.394	0.944 ± 0.083
Loch Sween	0.102		0.948 ± 0.160
Conwy	0.058	0.053	

Table 5.04 Estimates of genetic similarity and distance between three populations of *Ostrea edulis*. Values above the shaded diagonal are estimates of genetic similarity, those below are estimates of genetic distance (Nei, 1972).

	E			
	Solent	Loch Sween	Conwy	<i>C. gigas</i>
MDH	7.21	4.53	5.80	-
ICD	4.44	4.98	4.50	-
DIA	6.66	8.54	5.48	-
SOD	0.44	0.22	0.50	-
PGM	9.09	9.11	15.39	-
EST	2.05	-	-	2.22
GPI	1.43	1.51	0.91	0.91

Table 5.05 Estimates of the departure from evenness (E) of allele frequencies (Johnson & Feldman, 1973) for three populations of *Ostrea edulis* and for *Crassostrea gigas*.

5.4 DISCUSSION

5.4.1 Genetic Variation

The primary aim of this electrophoretic study was to complement the quantification of physiological responses with an examination of genetic similarities and differences in the populations of *Ostrea edulis*. The investigation also allowed further comparisons to be drawn with other studies of *O. edulis* and other species of bivalves, as a means of quantifying differences between populations, and establishing whether those differences occurred at the genotypic or phenotypic level. Although this study did not include as many populations or enzyme loci as some, it did highlight how similar these British populations are, both to each other and to some populations studied around Europe and the world.

In the present study, allozyme investigation showed most activity at the PGM locus, as was the case in the study of Johannesson *et al.* (1989). Other major banding activity was found at the DIA and ICD loci. The mean number of alleles per locus (table 5.02) indicated that Loch Sween animals have the greatest number, although there was no significant difference between the three populations. This would suggest this population has the greatest chance of allelic substitutions occurring and consequently would be expected to show the highest level of genetic variation. However, when the polymorphism values were compared, the values for the Solent and Loch Sween populations were almost identical, i.e. the increased number of alleles per locus had no significant effect in the degree of genetic variation observed. In a study of three non-sibling species of *Ostrea*, Buroker (1982) obtained a polymorphism value of 0.276 for *O. edulis* that compares very well with the values of 0.267 and 0.263 for the Solent and Loch Sween populations respectively. Other examples of polymorphism values are given in table 5.06. This table shows *Ostrea edulis* to be a species with less genetic variation than the other species. Only *Tiostrea chilensis* shows less polymorphism than *O. edulis*. In contrast *Crassostrea gigas* and *C. angulata* demonstrate greater than 50% of their loci are polymorphic. As a comparison, the gastropod *Littorina* shows polymorphism levels similar to some of the oysters. It may be assumed from this relative lack of genetic variation demonstrated by *O. edulis* that any differences in disease resistance, immunology and physiology are a result of

phenotypic adaptation to the environmental conditions.

5.4.2 Heterozygosity

Direct estimates of heterozygosity were not made in this study since Lessios (1992) has indicated that tests for Hardy-Weinberg expectations should be confined to testing a specific hypothesis based on the biology of the population, or on the genetics of a specific locus. Such tests should only be carried out for this reason, and when they are employed in extensive fashion with no intention of testing any particular hypothesis, they are of limited value. There are also a number of other problems which arise when this system of analysis is used. Non-significant results may imply that the population is in normal Hardy-Weinberg equilibrium, but this would not detect certain factors. A locus would not be evident that was undergoing strong selection but which actually had no appreciable deviation from Hardy-Weinberg proportions. Equally, factors which cause change from Hardy-Weinberg expectations to alter genotype frequencies in opposite directions, giving a non-significant result, would also not be evident. If significant effects were found, then one or more of the pre-requisites for Hardy-Weinberg equilibrium does not hold, but since Lessios (1992) lists at least ten of these it would be very difficult indeed to interpret the results. For these reasons polymorphic values were used as the estimates of genetic variation within populations.

The pairwise examination of homologous loci gave an estimate of the evolutionary divergence between the geographical populations of *Ostrea edulis*. Figure 5.03 shows that these populations were 90-100% genetically similar at nearly three quarters of their loci, with the decrease in similarity occurring mainly at the GPI and ICD loci (table 5.03). This gave an overall genetic identity of 0.929 ± 0.208 . Buroker *et al.* (1979a; 1979b) examined geographically isolated populations of *Saccostrea commercialis* and *Crassostrea gigas* and discovered similar levels of genetic identity; the former species had an identity of 0.992 ± 0.007 and the Pacific oyster an identity of 0.988 ± 0.016 . However, as populations become increasingly genetically isolated, as in the sequence of events

described in section 5.1.2, the genetic similarity between the populations decreases. This was shown by Buroker (1983) in three non-sibling species of *Ostrea* which had an overall genetic identity 0.245 ± 0.068 . As the three populations of this study become more in-bred, and there is a continued embargo on the movement of oysters between sites, it would be expected that their genetic identity would decrease through increased opportunity for inbreeding.

Species	Polymorphism Value	Reference
<i>Ostrea edulis</i>	0.182 - 0.409	Saavedra <i>et al.</i> (1993)
<i>Tiostrea chilensis</i>	0.172	Buroker <i>et al.</i> (1983)
<i>T. lutaria</i>	0.276	
<i>Crassostrea gigas</i>	0.583 - 0.630	Buroker <i>et al.</i> (1979a; 1979b)
<i>C. angulata</i>	0.600	
<i>Saccostrea commercialis</i>	0.464	
<i>S. glomerata</i>	0.519	
<i>Littorina rudis</i>	0.333 - 0.429	Ward and Warwick (1980)
<i>L. arcana</i>	0.519	

Table 5.06 Polymorphism values for different molluscan species

The large standard deviations for this study reflect the smaller sample size compared with other studies. This also caused some of the spurious results in table 5.02, with I values greater than one. However, table 5.02 indicated that at the SOD locus the Solent and Conwy populations were genetically identical. The lack of differentiation at this locus, the similarities at three others, and the increased

differentiation at another (DIA) suggest that any selective pressures which are present either at Conwy or in the Solent, are only acting at certain parts of the animals' enzyme system; this is discussed more fully in section 5.4.4. If there is any selective pressure occurring and it affects the oysters' metabolic system, then this could lead to the physiological differences described in chapter 3. It is possible that by influencing the effectivity of metabolic pathways a more efficient means of energy utilisation and/or extraction is promoted, resulting in differences in energy partitioning. However, the lack of significant difference in the mean similarities between the populations suggests there is little genetic influence on metabolic pathways, and that these differences arise by physiological adaptation to the environment. The genetic basis for these kinds of difference is discussed more fully in section 5.4.4.

There may also be 'founder effects' whereby the founders of a new population, e.g. those introduced to Conwy from the Solent, only have a fraction of the genetic variation of the parental population. This would be exhibited in the variations observed with one genetically identical locus and one highly differentiated. When overall genetic similarities were considered (table 5.04) it was seen that the Solent population was much more closely related to Conwy than to Loch Sween, reflecting the parental stock status. Paradoxically the Welsh and Scottish populations were more closely related to each other than to the English, but with some variation (statistically there was no significant difference between the three *I* values). This reflected the low levels of genetic variation which have been reported for *O. edulis* (Johannesson *et al.*, 1989; Saavedra *et al.*, 1993), and may be a product of their mode of development and reproduction. Buroker (1985) studied genetic variation in different members of the Ostreidae family and discovered that traits involved with reproduction (e.g. mode of larval development, fecundity, egg size, planktonic larval period, and initial planktonic larval size) co-varied with lower levels of genetic variation in larviparous species such as *O. edulis*. Oviparous (egg releasing) species, such as *Crassostrea*, have reproductive traits which co-vary with high levels of genetic variation. These life history and genetic variables reflected a greater variety of evolutionary patterns amongst the brooders than amongst the non-brooders, and would suggest that the larviparous

group have the greater opportunity to evolve under a wider variety of ecological conditions than oviparous ones, reflected in that they are found from the inter-tidal zone to water depths $\geq 200\text{m}$.

The genetic identities for *O. edulis* and *C. gigas* comparisons (table 5.03) appear very variable. The similarity observed at the EST locus was also found in the study by Buroker *et al.* (1975); the lower values of I for the GPI locus reflect the evolutionary divergence between the two oyster genera. Further investigation is needed into the genetic variation in *Crassostrea* species; the identification of specific areas of variation could then be applied to the successful culture of the mollusc. By using different alleles as biological markers particular strains of artificially bred oysters can be reared.

5.4.3 Effect of Loci on Growth Rate

As described before, heterozygosity has an effect on metabolism (Koehn and Shumway, 1982), fecundity (Rodhouse *et al.*, 1986), and growth rate (e.g. Garton *et al.*, 1984), but these and many other studies have only employed a small number of loci for examination. This study only found activity at seven of the ten loci used, but this is still more than the modal (5) and mean (6) number of loci used in 39 studies reviewed by Koehn *et al.* (1988). These authors showed that locus specific effects of heterozygosity on growth rate differed, and, depending on which loci were used for the comparison, different levels of correlation could be observed. Of the fifteen allozymes they examined they discovered that, with two exceptions, significant effects of heterozygosity were produced by enzymes involved with protein catabolism, and pre-glycolytic or glycolytic functions, such as the LAP, PGM and GPI loci in this study. Other enzymes involved with different metabolic functions, such as redox balance or detoxification, showed no significant effects on growth rate (for example the ICD and SOD loci of this study). One of their exceptions was GPI which had no significant effect on growth rate. Table 5.03 indicated that the GPI locus had the greatest genetic distance between the Solent and Loch Sween populations; it may have been assumed that this locus could have contributed to the differences observed in energy balance

between the populations but this appears to disagree with the results of Koehn *et al.* (1988). Likewise larger genetic distances were obtained for SOD which is an enzyme described as having no significant effect on growth rate (Koehn *et al.*, 1988). Those enzymes which are normally associated with heterozygote differences in growth rate, such as PGM and ICD, had very high degrees of genetic similarity, suggesting there would be no genetic basis for differences in physiological phenotype. These data lead to the conclusion that there is little or no genetic basis for observed physiological differences in the three British populations.

Koehn *et al.* (1988) went on to state that environmental and/or genetic factors other than the studied loci affect growth. These factors may act directly on the animals' energetic balance (for example changes in quantity and quality of food), or they may affect particular enzymes or loci which then effect the animal metabolism. Environmental salinity changes have been shown to affect the total AP-1 enzyme activity in *Mytilus edulis*, with populations under oceanic salinity having 50% greater levels of specific activity than animals under estuarine conditions (Koehn *et al.*, 1980). Acclimation from 33‰ to 15‰ gave a decrease in enzyme activity (not concentration) after nine days, and *vice versa*. Hazel and Prosser (1974) showed that high temperatures elicit an increased enzyme protein concentration in digestive enzymes, which may confer a fitness advantage under temperature stress. Other authors have suggested that heterozygote and growth rate correlations may be influenced by environmental stress (Koehn and Shumway, 1982; Diehl *et al.*, 1986; Gaffney, 1986). Buroker *et al.* (1983) however showed that animals of the same genotype could be subject to morphological changes depending on the physical environment. Three 'ecomorphs' of the New Zealand dredge oyster *Tiostrea lutaria* represent responses of the same genotype to different environments. When morphs were transplanted between environments, they were later seen to develop the characteristic phenotype of that environment.

In trying to establish any genetic basis for differences in physiology, energy balance and metabolic maintenance costs the direct effect of environmental fluctuations on individual growth patterns must always be taken into account (Hawkins *et al.*, 1993c). Bayne (1976) established that individual energy balance

influences individual growth rates and reproduction in pelecypods. Subsequently the effects of heterozygosity on growth rate (Zouros *et al.*, 1980; Garton *et al.*, 1984) and energy balance (Koehn and Shumway, 1982; Hawkins *et al.*, 1986) have been shown, which has led to research into the understanding of physiological and genetical processes involved in bivalve energetics (Koehn and Bayne, 1989). The present electrophoretic study suggests there are no significant heterozygotic differences between the three populations, even though physiological differences exist (chapter 3).

5.4.4 Effect of Sample Size

Further explanation of the lack of correlation between genotype and phenotype may be gained from examining the sample size and stage of development for those animals used in this study. The number of animals used was limited by the time in which the study had to be made. Repeated observations were made and a wide number of loci were tested to try to make up for this, but the results obtained were found to be statistically non-significant as a result of sample size. However, there is no other investigation which attempts to use such a comprehensive suite of physiological, immunological, metabolic and genetical measurements and this more than justifies the comparisons made. The animals were tested as a comparative measure in order to detect any genetic differences which occurred between the populations, and not to quantify specific genetic effects on energy budgets. For this reason the animals were sampled from their respective sites at the same time of year but without looking at specific stages of growth or reproduction. Animals of the same age and size class were used as it is well documented that somatic growth and gonad development can effect estimates of heterozygotic effects on growth rate and fecundity (Rodhouse *et al.*, 1986; Zouros *et al.*, 1988; Koehn, 1990; Zouros, 1990). Thus the similarities observed, although not necessarily statistically significant, are real and not artifacts of age or developmental stage in the sampling.

5.4.5 Summary

The determination of the physiological and genetical effects on energetics in bivalves is a complicated and often contentious subject (*vide* Koehn, 1990; Zouros, 1990). Whilst growth, growth rate and net energy balance are all functions of the environment, the specific consequence of the environment on energy balance also depends on the individual genotype (Koehn and Bayne, 1989). The electrophoretic study of three British populations has shown them to be genetically very similar, with little genetic variation in the species, confirming other observations made on *O. edulis* populations (Buroker, 1983; Johannesson *et al.*, 1989; Saavedra *et al.*, 1993). However, although the SFG measurements (chapter 3) were made on the same three populations as these genetic estimates, direct comparisons linking genetic differences and physiological differences are very difficult to make, owing to the problems highlighted by size. It may be hypothesized that lack of genetic variability observed means that the differences in SFG between the populations are a result of phenotypic plasticity, giving rise to particular ecotypes.

CHAPTER SIX

MONTHLY SAMPLING OF *Ostrea edulis*

FROM THE SOLENT

CHAPTER 6

MONTHLY SAMPLING OF *Ostrea edulis* FROM THE SOLENT

6.1 INTRODUCTION

The dynamic nature of marine and estuarine environments has considerable influence on the existence of resident organisms. Sessile bivalves are especially susceptible to changes, and their inability to move between locations exposes them to fluctuations in conditions from favourable to potentially lethal. Laboratory studies can provide information on a few parameters which influence the existence of a species, but the multiplicity of interactions which occur in the natural environment can only be established and quantified by repeated sampling of the animals. The use of field measurements of diseases in populations, together with concurrent laboratory based control studies, was recommended by Sparks (1993) as this method provides baseline data at the same time as possibly establishing how or why a particular phenomenon occurs, and so this procedure was used for the present study.

6.1.1 Field Investigations in Marine Bivalves

For this study, a monthly sampling regime was established to measure seasonal changes in an *Ostrea edulis* population of the Solent. A previous study of these oysters (Hawkins and Hutchinson, 1990) had already monitored immunology over a period of eighteen months, and provided evidence of a link between a seasonal change in water quality, in the form of a bloom of the ciliate *Mesodinium rubrum*, and large scale changes in oyster immunocompetence (Crawford *et al.*, 1993). An increase in particulate numbers was measured in the Solent, with a size spectrum comparable with that of bacteria and this was cited as the cause of the

immunological changes observed, as increased loads of *Vibrio* bacteria have been associated with *Mesodinium* blooms (Romalde *et al.*, 1990). *Vibrio anguillarum* was therefore used as a model pathogen to gauge the seasonal immunocompetence of *O. edulis*.

6.1.2 Physical Characteristics of the Solent

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 varies between 10 and 20 metres although there are shallow areas, such as the Brambles Bank, and isolated deeper areas. The west Solent (figure 6.01) is 2 nautical miles wide compared with 3 nautical miles for the east Solent (Key and Davidson, 1981; Christian, 1987). Although the Solent regime is affected by the tidal characteristics of the English Channel, the shallower water, bottom topography and land boundaries of the region also affect the tides. The Solent has very complex tidal regime, the most prominent feature of which is the double high water of Southampton Water. The ebb tide has a relatively short duration (3.75 hours) creating an ebb tidal velocity which is up to 100% greater than the corresponding flood velocity (Associated British Ports tide tables for Southampton). The tidal range in the west Solent is greater than in the east Solent, as are the tidal stream velocities. The Admiralty tidal stream atlas shows that these velocities can reach 3.5 knots (1.8 m s^{-1}) in the west Solent at spring tides.

The general airstream is from a westerly direction (Pingree, 1980) and the flow in the English Channel responds quickly to wind changes, within a day or so. When the winds are added vectorially the resulting direction in the summer is westerly, with a speed of about 2.5 m s^{-1} . In winter the winds are generally from the west-south-west with a speed of approximately 5 m s^{-1} , and thus the residual water flow through the region is greatest in winter. The Sowley fishing ground (figure 6.01) is part of the west Solent available for public harvesting of oysters, and consists of large areas of clear gravel with smaller areas of *Crepidula fornicata* banks (Key and Davidson, 1981). The outer edge of the bank has many larger stones, heavily encrusted with barnacles.

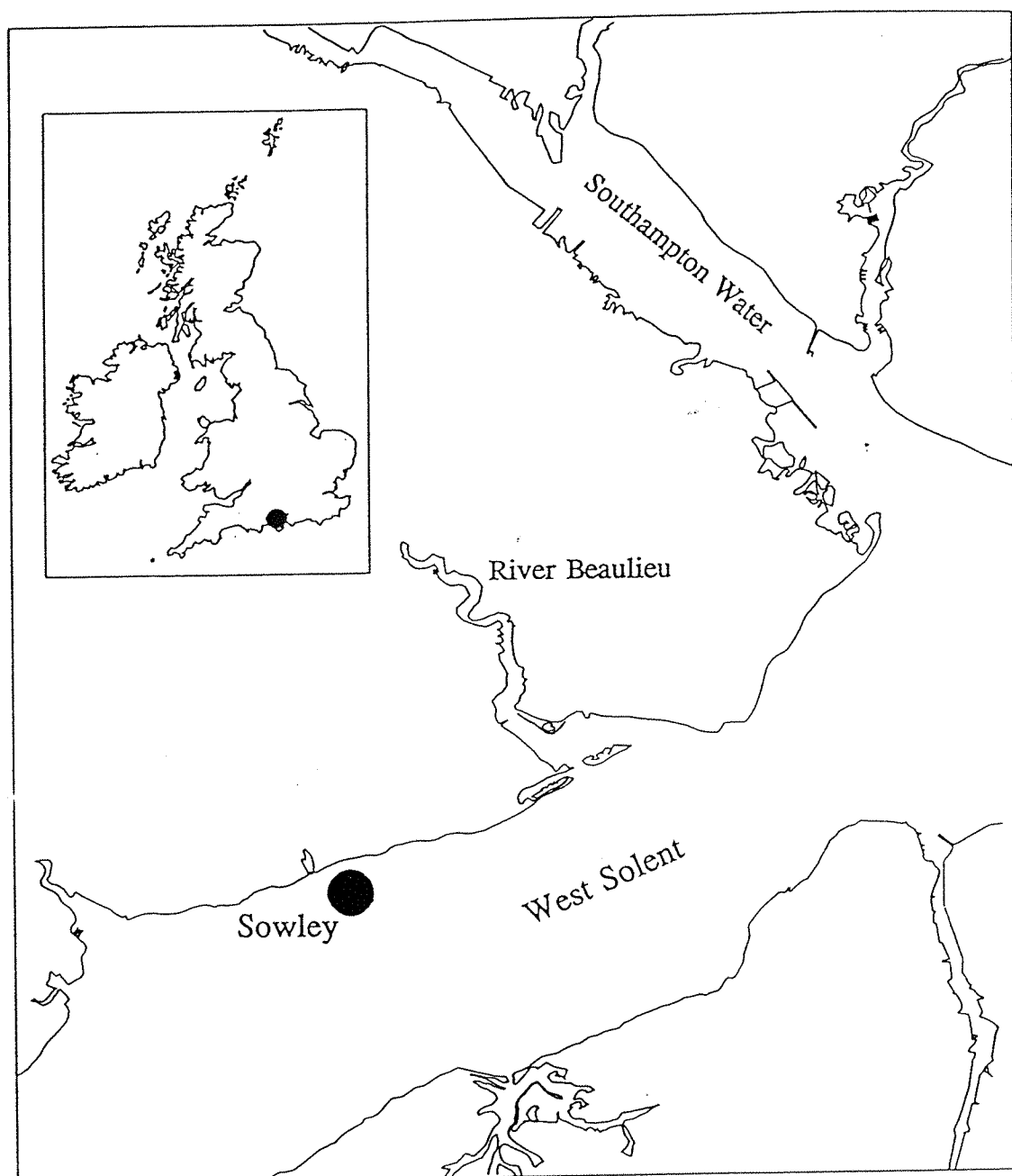


Figure 6.01 Position of Sowley sampling site in the Solent.

6.1.3 Immunological Indices

The molluscan immunology system relies on both haemocytic and haemolymph parameters. Phagocytic haemocytes participate in a variety of important physiological functions, for example defense against pathogenic invasion, wound repair, and nutrient transport. Consequently, anything which has a deleterious effect, such as heavy metal or hydrocarbon toxicity, environmental change, or outbreak of disease, will impair the functioning of these processes (Cheng and Sullivan, 1984; Cheng, 1988). The process of phagocytosis and the use of phagocytic indices in bacterial challenge of molluscan haemocytes has been extensively researched and is discussed later.

Haemolymph characteristics of molluscan immunology consist primarily of microcidal agents elicited by respiratory burst activity, and the generation of lysosomal enzymes. The former method includes hydrogen peroxide production, as found in the haemolymph of *Patinopecten yessoensis* (Nakamura *et al.*, 1985), *Lymnaea stagnalis* (Dikkeboom *et al.*, 1985), *Biomphalaria glabrata* (Shozawa, 1986), *Pecten maximus* (Le Gall *et al.*, 1991) and other molluscs, as well as the oysters *Ostrea edulis* and *Crassostrea gigas* (Hawkins and Hutchinson, 1990; Bachere *et al.*, 1991). Lysozyme, and other lysosomal enzymes such as β -glucuronidase and aminopeptidase, have been demonstrated both intra- and extra-cellularly in *M. mercenaria*, *B. glabrata*, *C. virginica*, *C. gigas*, *O. edulis* and other molluscs by a variety of authors (McDade and Tripp, 1967; Feng *et al.*, 1971; Rodrick and Cheng, 1974; Cheng and Rodrick, 1975; Cheng, 1978).

6.2 MATERIALS AND METHODS

6.2.1 Collection and Maintenance of Animals

Ostrea edulis were collected every four to five weeks from the public fishing grounds at Sowley in the west Solent (figure 6.01); a very late sampling in July 1993 was used for both July and August measurements, and bad weather prevented sampling in November 1993, resulting in sixteen monthly samples being taken between June 1992 and December 1993. The animals collected were transported in water to the Department of Oceanography, where they were transferred to a flow-through aquarium. Conditions were controlled by micro-processor with the temperature and salinity adjusted to the same as field conditions (6.7-18.8°C and 32.2-36.5‰). Animals were kept for a minimum of three days in these conditions before any assays were carried out. The oysters were fed with a mixed algal ration of the flagellates *Tetraselmis suecica* and *Isochrysis galbana*, and the diatom *Phaeodactylum tricornutum*, supplied slowly (approximately 5-10 ml min⁻¹) to the tank by a peristaltic pump.

6.2.2 Field Measurements

Water samples were collected from the surface only, as a previous study showed surface and bottom samples to be almost identical for all physical characteristics (Hawkins and Hutchinson, 1990). A 10 litre sample was collected each month and transported back to the Department of Oceanography in a polythene carboy. When necessary, the water sample was stored overnight in a 5°C constant temperature room, although the sample was usually processed the same day as collection.

Temperature and salinity were measured *in situ* at the Sowley sampling site, using a custom-built, continuous reading salinometer. In the laboratory, 500 ml of the water sample was filtered through a pre-ashed Whatman™ GF/C filter, and then dried (at 70°C) and ashed (at 500°C), to give a measure of the organic and inorganic fraction of the total particulate load (mg l⁻¹).

Five sub-samples of the water sample were processed through a Coulter™ TAPI electronic particle counter, using a 2 ml volume and a 200 µm aperture tube,

to give a size spectrum analysis of the suspended sediment load. This also provided information on modal particle size.

6.2.3 Phagocytic Index

Vibrio anguillarum bacteria were used to provide a pathogen challenge quantifiable by haemocytic and immunological indices. The use of an index of phagocytosis gives an indication of large granulocyte mobility and sensitivity to a pathogen under different seasonal conditions. The bacteria used for the assays were in a nutrient broth solution, but were maintained in both broth and on solid agar media. The methods for this are given in Appendix A3.

An oyster was removed from the aquarium and opened. Approximately 500 μ l of haemolymph was taken, using a drawn out pasteur pipette, from the adductor muscle blood sinus. A 'bacterial broth' was prepared by filtering aquarium sea water through a Whatman™ 0.1 μ m cellulose nitrate filter; 0.05-0.1 % (w/v) of primulin (SIGMA), a vital stain, was dissolved in 10 ml of this filtered sea water. 100 μ l of the primulin solution was then mixed with 100 μ l of bacterial broth. A 100 μ l sub-sample of the haemolymph was placed in an embryo dish and mixed with 100 μ l of the primulin-stained bacterial broth. 50 μ l of this mixture were placed on a microscope slide, and a cover-slip added carefully. This sample was allowed to incubate for one hour at room temperature before examination, allowing phagocytosis to occur, and haemocytes and bacteria to take up sufficient vital stain. The slide was examined under a Zeiss Standard Universal microscope with a model II F1 vertical illuminator for fluorescence; barrier filters (53,0,0) and excitation filters (BG 38/2.5) were also used resulting in amoebocytes and bacteria fluorescing bright yellow. The large granulocytes were observed and identified, and bacteria taken up by these cells were counted. A minimum of six oysters were sampled this way each month, and a minimum of twenty amoebocytes were identified and associated bacteria counted, for each month except for December 1993 when only seventeen amoebocytes were identified. The protocol for this procedure is summarised in figure 6.02.

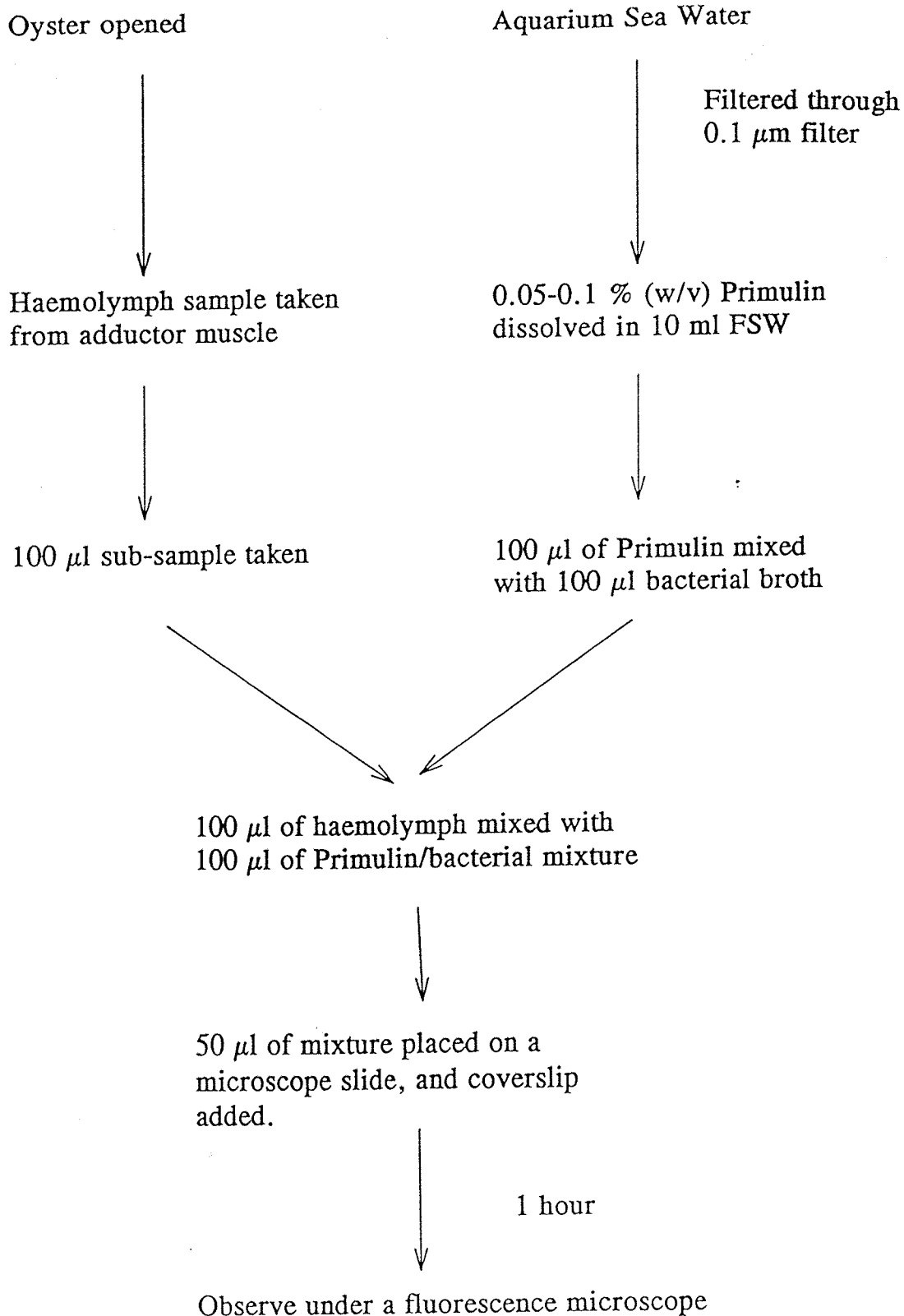


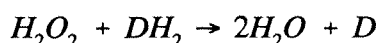
Figure 6.02 Protocol for fluorescent analysis of phagocytic uptake of bacteria.

6.2.4 Immunological Measurements

Hydrogen peroxide concentration and lysozyme activity were measured monthly, to indicate natural variability - both assays were performed per oyster haemolymph sample. A pathogen challenge was used as a means of quantifying seasonal changes in disease susceptibility, but these samples were different from the samples taken for the phagocytic index.

a) Hydrogen Peroxide Determination

This was done spectrophotometrically, based on the method described by Meiattini (1984). It is possible to determine the peroxide concentration of a given sample by using a suitable hydrogen donor which is colourless when reduced, but turns blue on oxidation. The reaction is given by :



For this determination the hydrogen donor (DH_2) is 4-aminophenazone / chromotropic acid. The intensity of the colour produced is proportional to the concentration of the hydrogen peroxide in the solution. The absorption coefficient varies with the experimental conditions, so the absorbance of the sample is compared with the absorbance of a standard solution.

Haemolymph samples were taken from the oysters and a sub-sample was de-proteinised immediately, as other peptides in the sample may have affected the assay - hydrogen peroxide is unstable in some protein containing solutions. To deproteinise the sample, 100 μ l of haemolymph was pipetted into a centrifuge tube together with 50 μ l of 6% perchloric acid. 50 μ l of 6M sodium hydroxide was added to this to neutralise the acid, but the resulting denatured protein formed a precipitate, so the sample was centrifuged at 3000 rpm for 2 minutes. 100 μ l of the centrifuged supernatant was then used in the assay.

Peroxidase reagent was made up as in table 6.01. The azide is present to prevent bacterial action and inhibit the action of catalase (which may be present in biological samples), and the Triton X-100 helps to solubilise any lipid fractions.

The reagent was kept in a stoppered, brown bottle at 0-4°C, and was made up fresh each month. The standard hydrogen peroxide solution was made up fresh for each assay, at a concentration of 20 mg l⁻¹. The absorbance of the samples was read on a CECIL CE292 spectrophotometer at a wavelength of 600 nm. 3 ml cuvettes were used with a light path of 10 mm.

2.5 ml of peroxidase reagent were pipetted into each cuvette and the initial absorbance was measured. 100 µl of hydrogen peroxide standard or deproteinised haemolymph sample was then added, with a stop-clock started simultaneously. Each sample was mixed with a glass rod, and the final absorbance was read after 30 seconds. The hydrogen peroxide concentration of the haemolymph sample was found by comparing the absorbance of the sample with that of the standard peroxide solution, multiplied by any dilution factor. This was given by :

$$\text{Peroxide Concentration } (\rho) = \frac{\text{Change in Sample Absorbance}}{\text{Change in Standard Absorbance}} \times 20 \times 2.1 \text{ mg l}^{-1}$$

Reagent	Quantity
di-Sodium Hydrogen Phosphate	2.67 g
Sodium di-Hydrogen Orthophosphate	1.726 g
Triton X-100	100 μ l
(Horseradish) Peroxidase Enzyme	0.004 g
4 - Aminophenazone	0.01 g
Chromotropic Acid	0.4 g
Distilled Water	100 ml

Table 6.01 Reagents used in peroxidase reagent for hydrogen peroxide determination.

b) Lysozyme Determination

This was also done spectrophotometrically, using the method described by Weisner (1984). This assay is based on the ability of lysozyme to lyse freeze-dried *Micrococcus luteus* in suspension at a known concentration. This reduces the turbidity of the sample which can be measured photometrically. The extent of reduction of turbidity is proportional to the catalytic activity of lysozyme in the assay mixture.

0.003 g of *Micrococcus luteus* lyophilisate was suspended in 15 ml of phosphate buffer, to give a concentration of 0.2 g l⁻¹. The phosphate buffer was made up with 8.05 g sodium di-hydrogen phosphate, 2.6 g di-sodium hydrogen phosphate, 0.9 g sodium chloride, and 0.52 g sodium azide, dissolved in 500 ml distilled water. This solution was adjusted to pH 6.3, and then diluted to 1000 ml. The phosphate buffer was stored at 0-4°C, and the *Micrococcus* suspension was freshly made up for each assay.

Absorbances were measured at 546 nm, with a light path of 10 mm. 1.5 ml of *Micrococcus* suspension was added to each cuvette; 25 µl of the haemolymph sample was added to the cuvette, and a stopclock was started simultaneously. The sample was mixed with a glass rod, and the absorbance was recorded after 30 seconds (A1), 120 seconds (A2), and 210 seconds (A3). The lysozyme activity was calculated by :

$$\frac{\Delta A}{\Delta t} = \frac{[(A1-A2) + (A2-A3)] \div 2}{\Delta t}$$

where $\Delta t = 90$ seconds.

The catalytic activity of lysozyme in each sample was given by :

$$X = \frac{Y - a}{b} \text{ Enzyme Units (E.U.)}$$

where $Y = \Delta A/\Delta t$, and a and b are constants, calculated from a standard curve, with $a = 1.16 \times 10^{-5}$, and $b = 2.874 \times 10^{-7}$.

c) Pathogen Challenge

Preliminary experiments were conducted to find the most suitable method for challenging haemocytes with *Vibrio anguillarum*. Initially, a sample of haemolymph was taken from the adductor muscle blood sinus, and a 400 μ l sub-sample of this was mixed with an equal volume of bacterial broth, before testing for hydrogen peroxide and lysozyme activity. However, it was found that chemicals in the nutrient broth interfered with spectrophotometric determination of peroxide, so this method was not used. Centrifugation of the bacterial broth, and re-suspension of bacteria in filtered sea water before challenging haemocytes, also proved unsuccessful as the bacteria appeared to lose their viability; this was tested by plating out the re-suspension on agar plates, which resulted in growth but only after two or three weeks.

The method eventually employed to challenge oysters with a bacterial pathogen resulted in animals being incubated individually in a closed body of water, to which was added the bacterial broth. Measurements of hydrogen peroxide and lysozyme were made after 1 hour, 6 hours, 24 hours, and 48 hours post-innoculation. The time-consuming nature of these assays meant a reduction in experimental group sizes, resulting in 3 experimental animals for each group from 1 hour to 48 hours, and a single control animal for each group. The T0 measurements were based on the monthly sampling group, where five animals were measured.

250 ml of seawater, at appropriate temperature and salinity conditions from field measurements, was put in each of sixteen Beatson jars. One control oyster was placed in each of four jars, and lids were secured. The remaining experimental jars had 100 μ l of seawater removed, and 100 μ l of bacterial broth added. A single animal was added to each jar, lids applied, and the prepared jars placed in a water bath at appropriate temperature for incubation. Hydrogen peroxide and lysozyme activity were then measured using the methods described above.

6.2.5 Statistical Analysis of Results

The results of laboratory experiments were expressed as mean values \pm standard error, unless otherwise stated. The number of observations at each point is specified above. Where appropriate data were analysed using an unpaired t-test (or a Mann-Whitney test as a non-parametric alternative) for comparing two conditions, or a standard ANOVA (or a Kruskal-Wallis test as a non-parametric alternative) for comparing more than two conditions. Two-way ANOVA was used to analyse temporal variation in monthly immunological measurements under pathogen, over a 48 hour period. Pearson product-moment correlations were performed to measure the association between variables; this test assumed the data was normally distributed and did not assign dependent and independent variables.

6.3 RESULTS

6.3.1 Field Measurements

The monthly variation in salinity (ppt) and temperature ($^{\circ}\text{C}$) at the sampling site are indicated in figures 6.03 and 6.04 respectively. Correlations for the different physical variables measured at Sowley are given in table 6.02..

Salinity showed a significant, positive correlation with temperature ($P = 0.002$), and a significant negative correlation with total particulate load ($P = 0.019$), total organic load ($P = 0.01$) and monthly lysozyme production ($P = 0.038$).

Temperature showed significant negative correlations with total particulate numbers, total particulate load, and total organic load ($P < 0.001$, for all comparisons).

The particle size spectrum in the suspended sediment load is represented in figure 6.05, and the total particle numbers are shown in figure 6.06. Calibrations for the Coulter™ Counter size channels are indicated in table 6.03. The suspended load of the late spring and early summer months consisted mainly of fine ($2.5\ \mu\text{m}$ diameter) particulate material. Particulate numbers increased in autumn and winter, with a 1992 peak in November, and the 1993 peak in December. The results for these months also showed an increase in the $3.18\text{--}8.00\ \mu\text{m}$ diameter particles. One-way ANOVA showed there were no significant differences between any of the months sampled ($P > 0.05$), indicating these changes in particulate load are probably a result of natural variability. Modal particle size (figure 6.07) further indicated that the majority of suspended load consists of fine material for most of the year. The influx of larger particles in winter is shown with increased diameters between November 1992 and March 1993.

The total organic load within the suspended sediment (figure 6.08) had a significant positive correlation with total particulate numbers and the total particulate load ($P < 0.001$, for both; table 6.02), and a significant negative correlation with temperature ($P < 0.001$) and salinity ($P = 0.01$). The inorganic fraction of suspended load (figure 6.09) showed no significant correlations with any of the other physical factors measured ($P > 0.05$). The organic and inorganic loads together make up the total particulate load (figure 6.10), which showed the same correlations as the organic fraction.

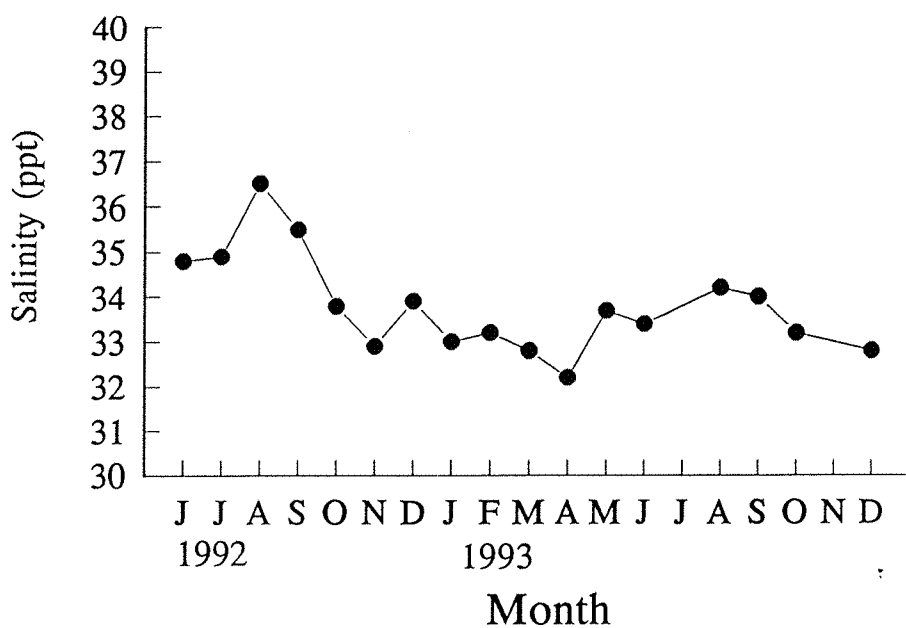


Figure 6.03 Monthly variation in surface water salinity (ppt) at the Sowley sampling site.

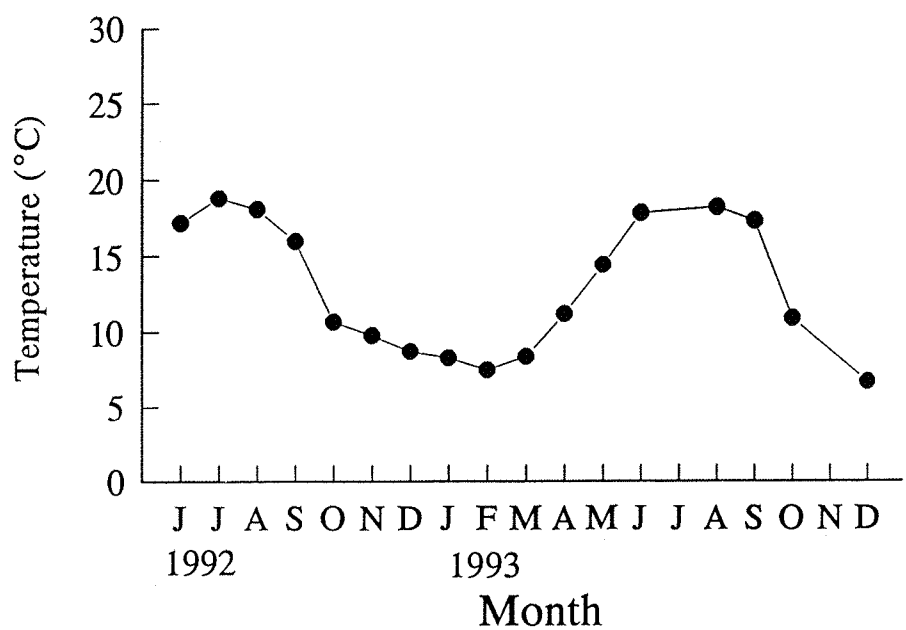


Figure 6.04 Monthly variation in surface water temperature (°C) at the Sowley sampling site.

	Lysozyme	Phagocytic Index	Total Particulate Numbers	Total Organic Load	Total Inorganic Load	Total Particulate Load (mg/l)	Temperature	Salinity
H ₂ O ₂	-0.007 <i>0.981</i> 16	0.101 <i>0.711</i> 16	0.153 <i>0.571</i> 16	0.464 <i>0.070</i> 16	-0.039 <i>0.886</i> 16	0.435 <i>0.092</i> 16	-0.404 <i>0.120</i> 16	-0.454 <i>0.077</i> 16
Lysozyme		0.022 <i>0.937</i> 16	0.040 <i>0.884</i> 16	-0.007 <i>0.979</i> 16	-0.383 <i>0.143</i> 16	-0.060 <i>0.827</i> 16	-0.341 <i>0.197</i> 16	-0.522 <i>0.038</i> 16
Phagocytic Index			0.046 <i>0.861</i> 17	0.189 <i>0.468</i> 17	-0.040 <i>0.880</i> 17	0.177 <i>0.497</i> 17	-0.136 <i>0.602</i> 17	-0.457 <i>0.065</i> 17
Total Particulate Numbers				0.816 <i>< 0.001</i> 17	0.240 <i>0.353</i> 17	0.817 <i>< 0.001</i> 17	-0.775 <i>< 0.001</i> 17	-0.447 <i>0.072</i> 17
Total Organic Load (mg/l)					0.228 <i>0.380</i> 17	0.992 <i>< 0.001</i> 17	-0.823 <i>< 0.001</i> 17	-0.605 <i>0.010</i> 17
Total Inorganic Load (mg/l)						0.346 <i>0.174</i> 17	-0.072 <i>0.784</i> 17	0.157 <i>0.547</i> 17
Total Particulate Load (mg/l)							-0.803 <i>< 0.001</i> 17	-0.563 <i>0.019</i> 17
Temperature								0.704 <i>0.002</i> 17

Table 6.02 Correlation of monthly figures for field measurements of the physical parameters at the Sowley sampling site. Cell contents represent the correlation coefficient (normal text), *P* value (italics), and the number of samples (bold), with significant comparisons shaded.

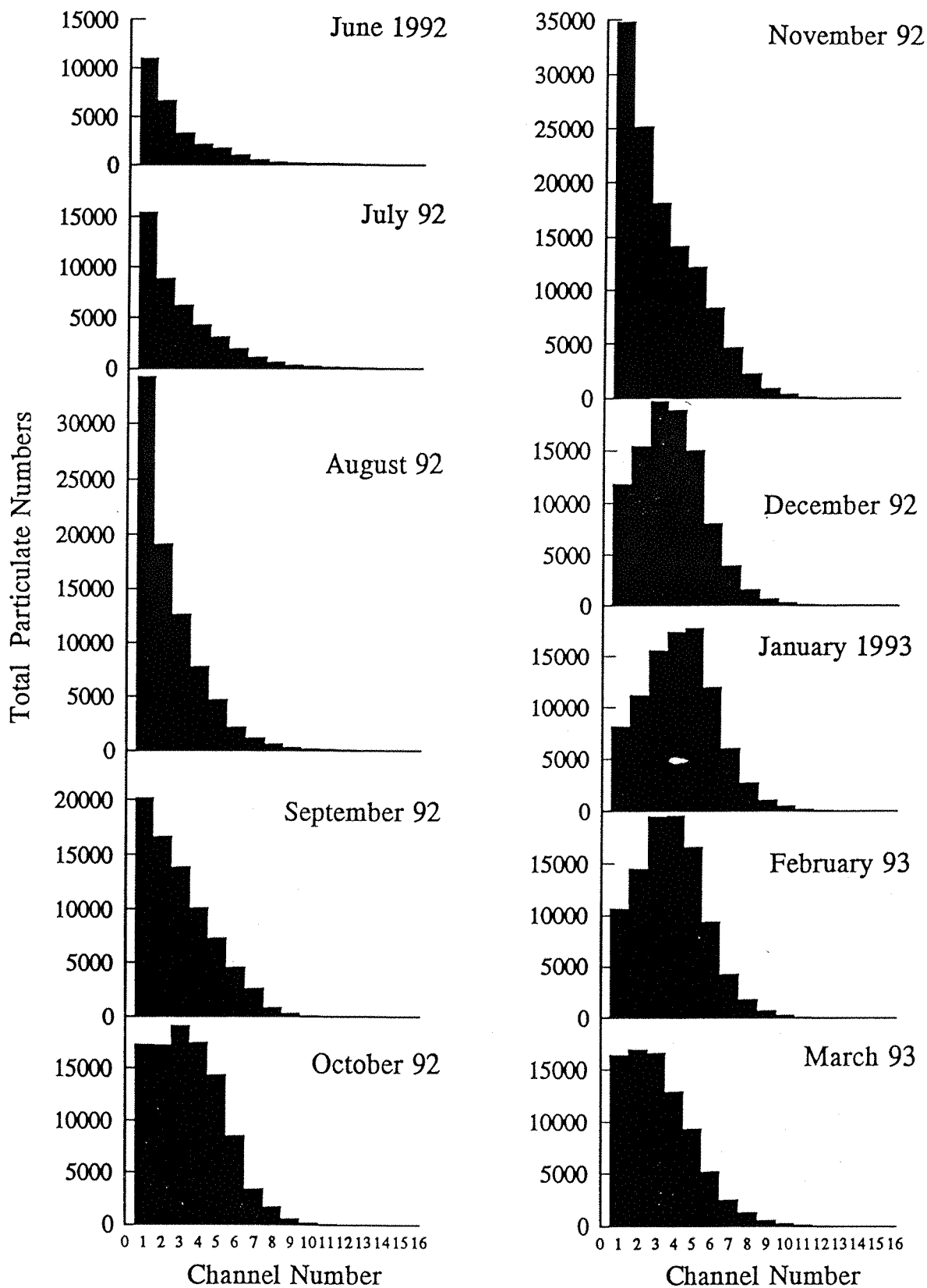


Figure 6.05 Monthly variation in distribution of particle size of surface water suspended sediment load from the Sowley sampling site. Sizes for the Coulter Counter channels are given in table 6.03. All samples = 2 ml volume.

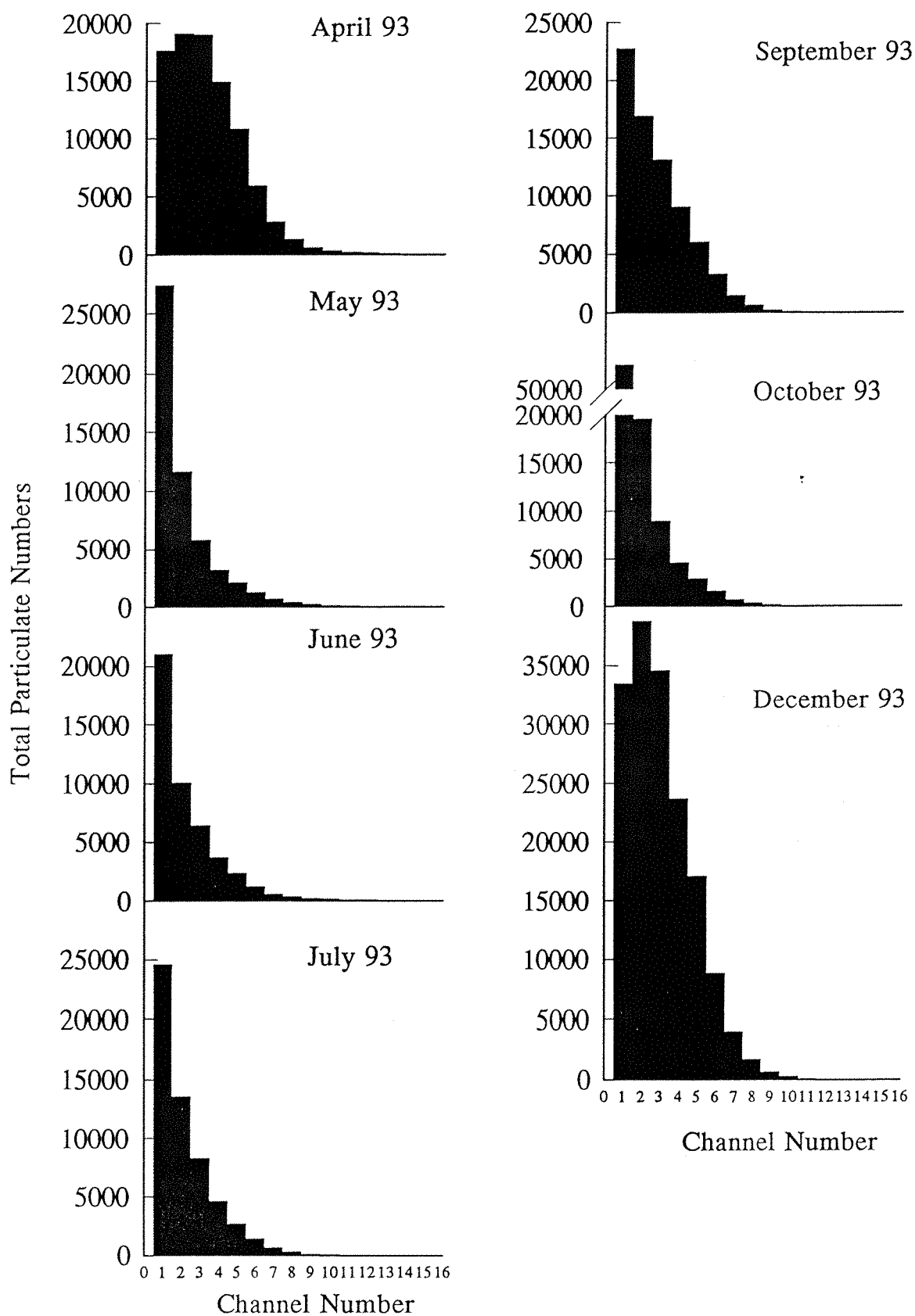


Figure 6.05 continued.

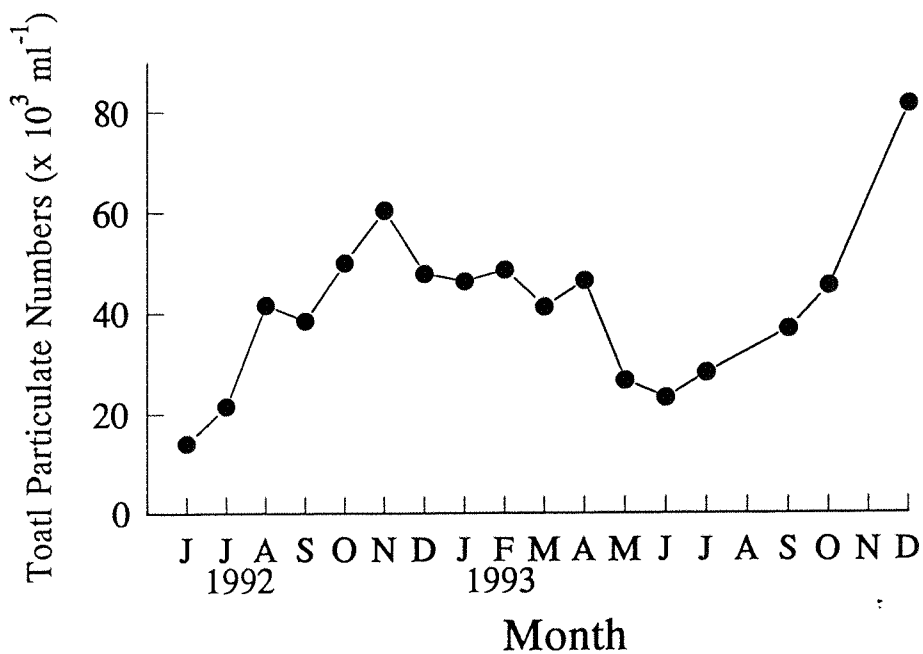


Figure 6.06 Monthly variation in surface water total particulate numbers ($\times 10^3 \text{ ml}^{-1}$) at the Sowley sampling site.

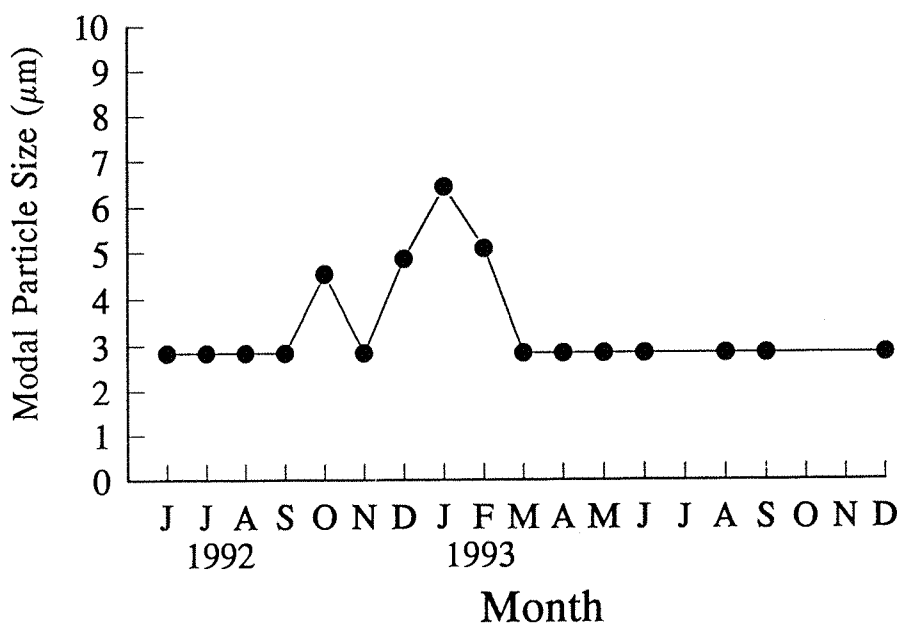


Figure 6.07 Monthly variation in the surface water modal particle size (μm) in the suspended particle load at the Sowley sampling site.

Channel No.	Size (μm)	Channel No.	Size (μm)
1	2.52	9	16.00
2	3.18	10	20.16
3	4.00	11	25.40
4	5.04	12	32.00
5	6.35	13	40.32
6	8.00	14	50.80
7	10.08	15	64.00
8	12.70	16	80.64

Table 6.03 Size channels used in Coulter™ Counter measurements of particulate counts.

6.3.2 Phagocytic Uptake of *Vibrio anguillarum* by Oyster Haemocytes

A phagocytic index of bacterial uptake by large granulocytes is shown in figure 6.11. No significant correlations were found between this index and any of the physical factors measured ($P > 0.05$). Results for one-way ANOVA tests of differences between the different months are given in table 6.04. There did not appear to be any regular pattern to the degree of phagocytosis occurring; the peak for 1992 activity occurred in early winter (November), whereas the 1993 peak occurred in summer (July).

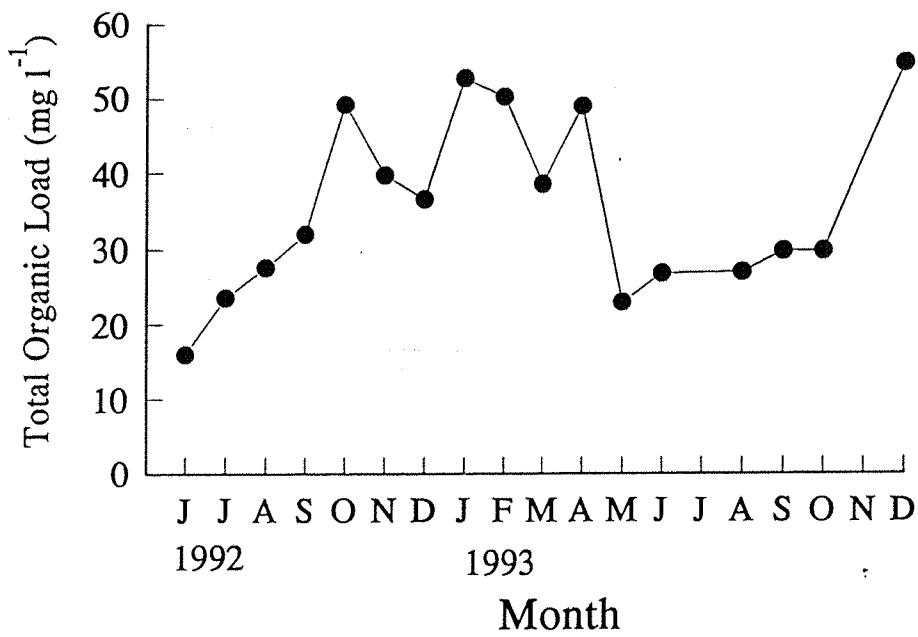


Figure 6.08 Monthly variation in surface water total suspended organic load (mg l⁻¹) at the Sowley sampling site.

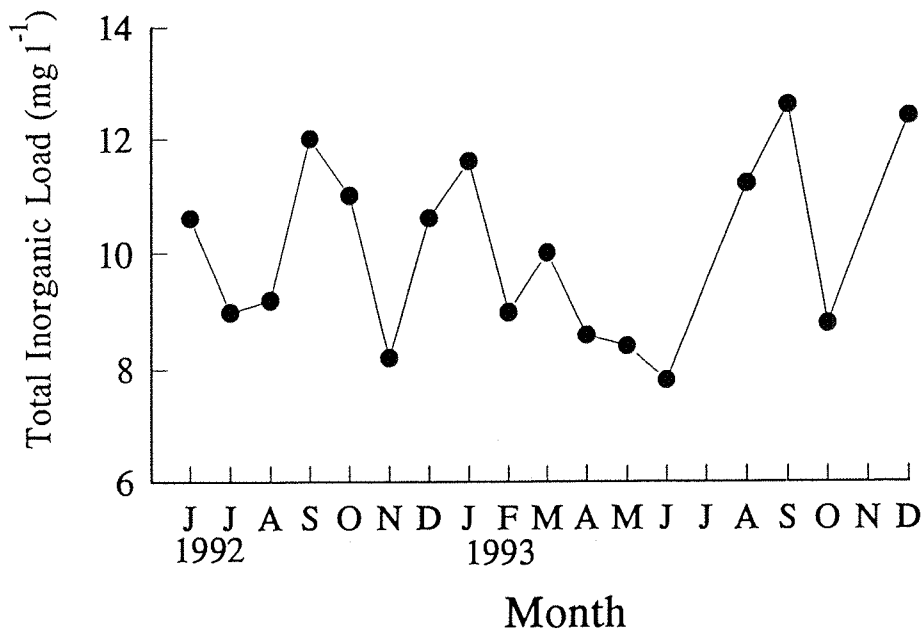


Figure 6.09 Monthly variation in surface water total suspended inorganic load (mg l⁻¹) at the Sowley sampling site.

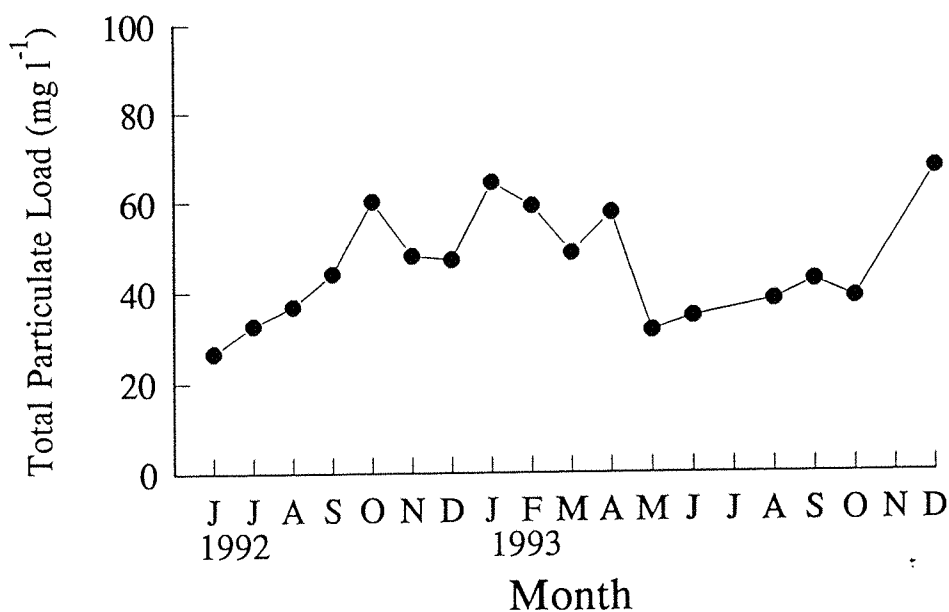


Figure 6.10 Monthly variation in surface water total particulate load (mg l⁻¹) at the Sowley sampling site.

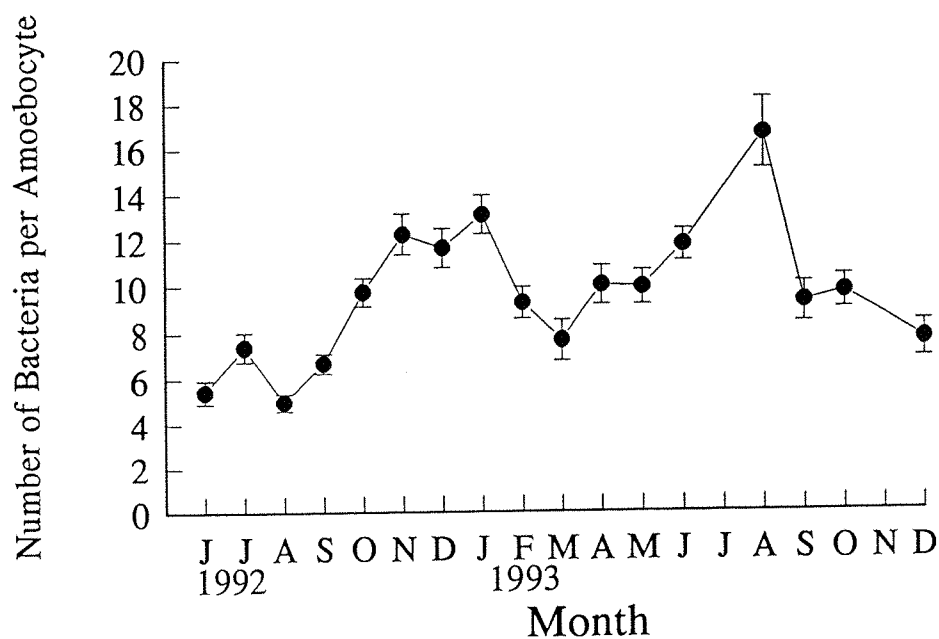


Figure 6.11 Monthly variation in the phagocytic index of numbers of bacteria taken up by amoebocytes (= large granulocytes) of *Ostrea edulis* at the Sowley sampling site. Mean ± SE, n ≥ 20 at each point, except December 93 where n = 17.

	J	A	S	O	N	D	J	F	M	A	M	J	J	S	O	D
Jun 92	n.s.	n.s.	n.s.	*	*	*	*	n.s.	n.s.	*	*	*	*	n.s.	*	n.s.
Jul		n.s.	n.s.	n.s.	*	*	*	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	n.s.	n.s.
Aug			n.s.	*	*	*	*	*	n.s.	*	*	*	*	*	*	n.s.
Sep				n.s.	*	*	*	n.s.	n.s.	n.s.	n.s.	*	*	n.s.	n.s.	n.s.
Oct					n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Nov						n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Dec							n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Jan 93								n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Feb									n.s.	n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.
Mar										n.s.	n.s.	*	*	n.s.	n.s.	n.s.
Apr											n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
May												n.s.	n.s.	n.s.	n.s.	n.s.
Jun													n.s.	n.s.	n.s.	n.s.
Jul														n.s.	n.s.	*
Sep															n.s.	n.s.
Oct																n.s.

Table 6.04 One-way ANOVA of monthly phagocytic activity. Shaded Boxes with * = significant difference ($P < 0.05$). n.s. = not significant

6.3.3 Haemolymph Immunological Measurements

Monthly haemolymph hydrogen peroxide concentrations are given in figure 6.12. There were no significant correlations between hydrogen peroxide production and the other physical parameters measured ($P > 0.05$), as with the phagocytic index. When multiple comparisons were made, using a one-way ANOVA, the only significant differences between months were found between September 1992 (the minimum value for that year) and January 1993 (the maximum for that year), and between January 1993 and August 1993 ($P < 0.05$). The variability encountered in these measurements eliminated any other significant differences ($P > 0.05$). However, despite the lack of significant differences or correlations, peroxide production appeared to increase during winter and fall to minimal levels in the late summer.

The monthly variation in lysozyme activity is shown in figure 6.13. A Pearson product-moment correlation showed that there was a significant, negative correlation between lysozyme and salinity ($P < 0.05$), but none of the other physical parameters had a significant correlation ($P > 0.05$). A one-way ANOVA of monthly lysozyme activity showed no significant differences between any of the months ($P > 0.05$), which reflected the high degree of variability observed in the results.

6.3.4 Effects of Pathogen Challenge on Oyster Immunology

Production of hydrogen peroxide over a 48 hour period, following bacterial challenge, is shown in figure 6.14. A two-way ANOVA indicated that there was no significant difference in peroxide concentrations from month to month, after allowing for the 48 hour period of each challenge experiment ($P > 0.05$). However, after allowing for the effects of monthly variation, significant differences in hydrogen peroxide concentration were found between T0 & T48, T1 & T48, and T24 & T48 ($P < 0.05$, for all comparisons). Mean concentrations of peroxide were calculated from these data, for each time period after the initial challenge, and these are shown in figure 6.15. Two-way ANOVAs for the control hydrogen peroxide concentrations indicated no significant effects over the 48 hour

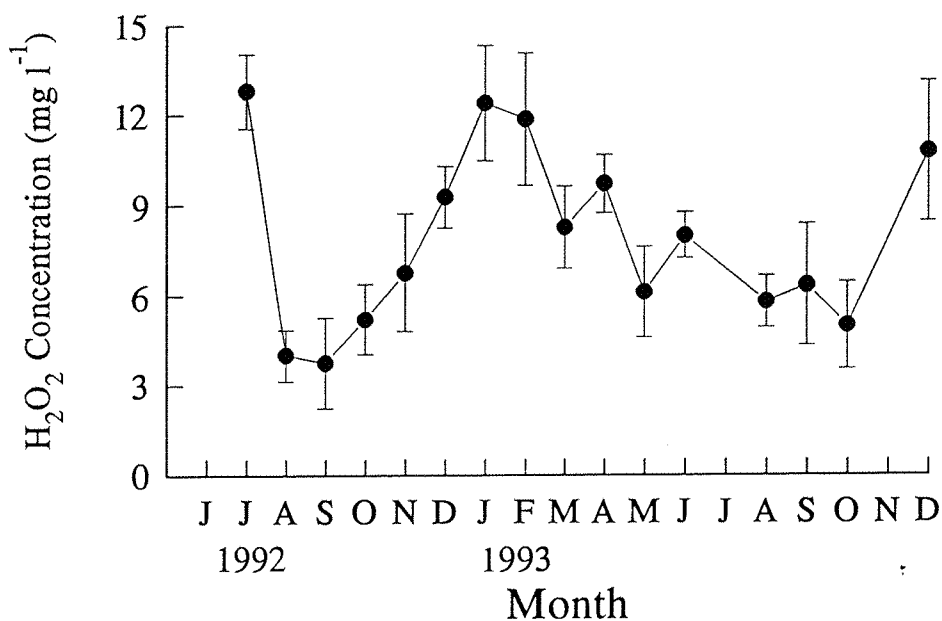


Figure 6.12 Monthly variation in the haemolymph hydrogen peroxide concentrations (mg l⁻¹) of *Ostrea edulis* at the Sowley sampling site. Mean \pm SE, n = 5 at each point, except June 92 where n = 4.

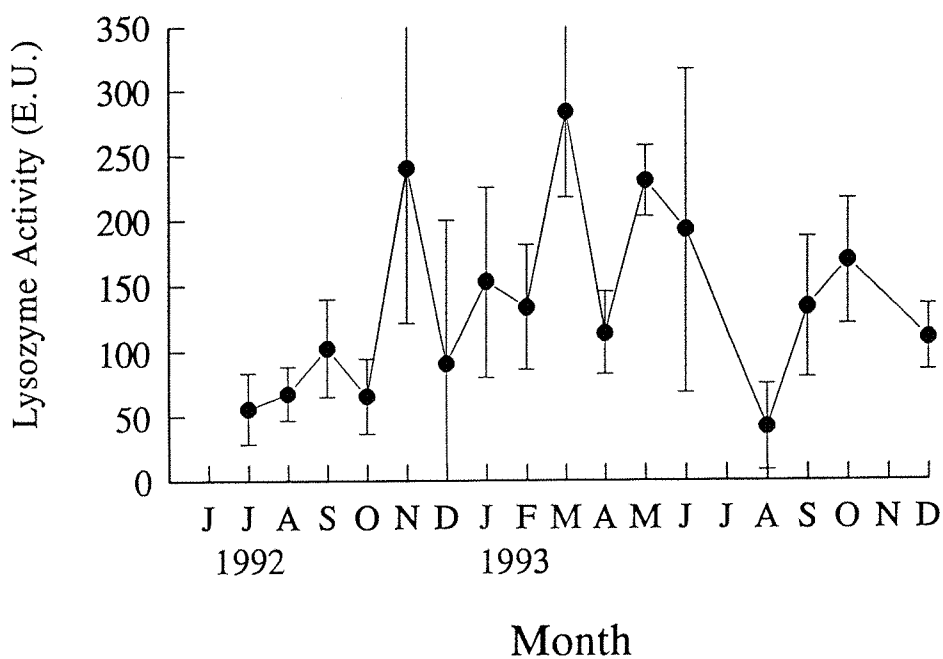


Figure 6.13 Monthly variation in the haemolymph lysozyme activity (enzyme units) of *Ostrea edulis* at the Sowley sampling site. Mean \pm SE. n = 5 at each point, except June 92 where n = 4.

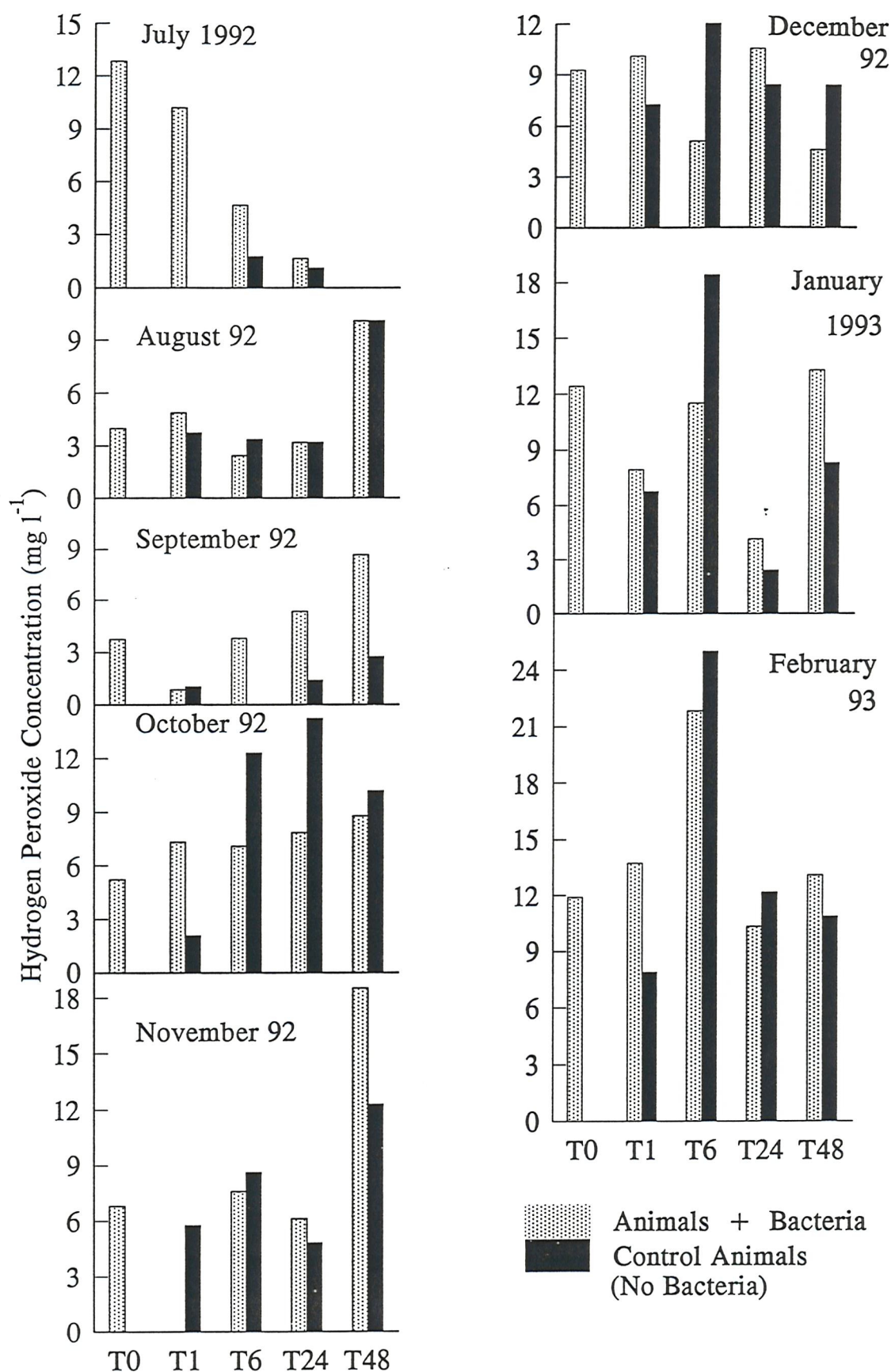


Figure 6.14 Monthly variation in haemolymph hydrogen peroxide concentrations (mg l^{-1}), following challenge with *Vibrio anguillarum*, of *Ostrea edulis*. Means, $n=5$ for T0, $n=3$ for T1 to T48, $n=1$ for controls.

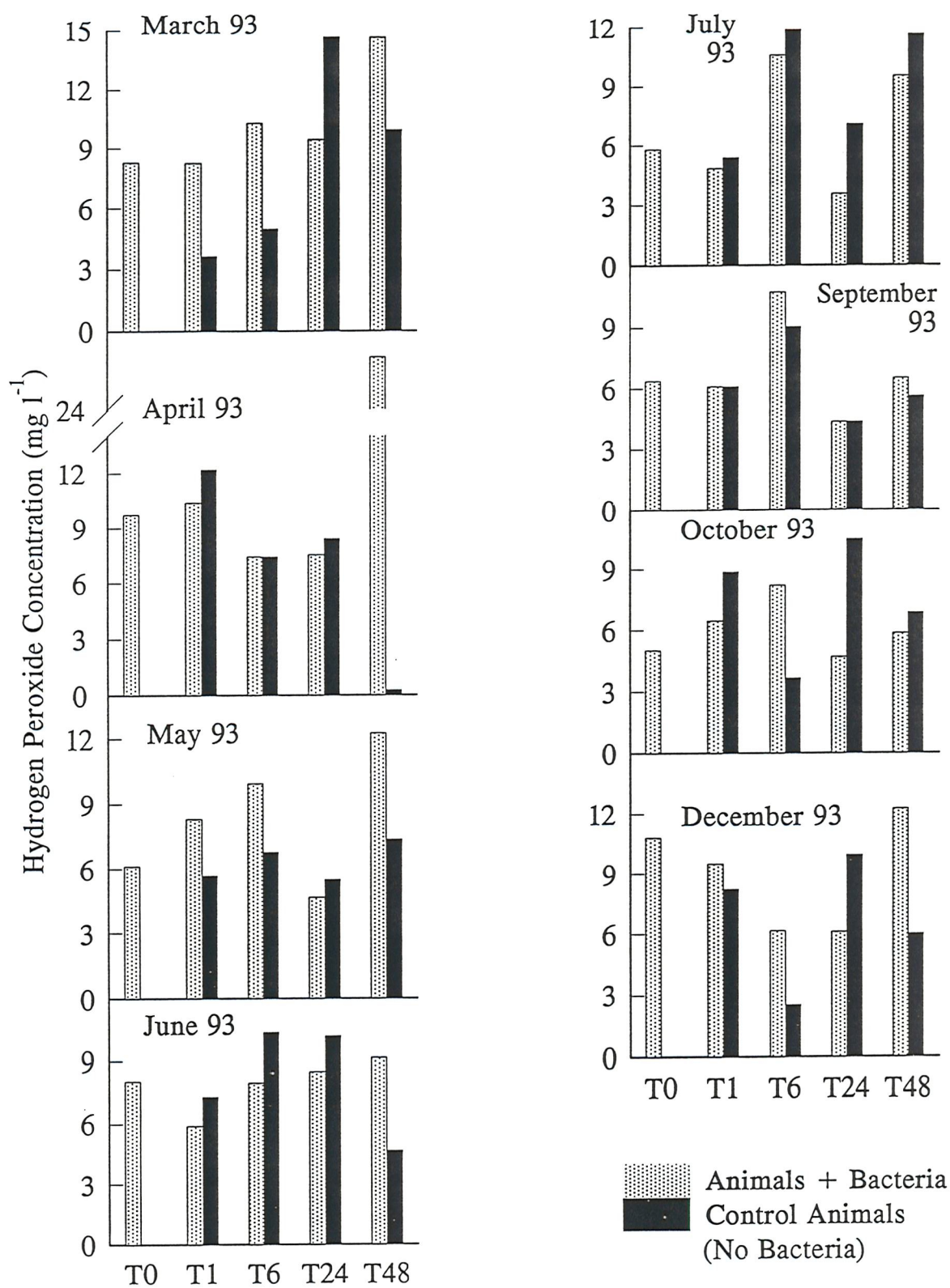


Figure 6.14 continued.

experimental period ($P > 0.05$), but showed some significant differences between February 1993 & July 1992, and February 1993 & September 1992 ($P < 0.05$), after other temporal factors had been taken into account. These months had missing data for some of the readings, which probably accounted for the statistical differences observed.

The data for lysozyme activity over a 48 hour period, following bacterial challenge, are presented in figure 6.16. Two-way ANOVAs for both experimental and control data indicated no significant differences between any of the months, or between any of the experimental readings ($P > 0.05$, for both). The mean values plotted for lysozyme against time (figure 6.17) allow comparisons to be made with the hydrogen peroxide patterns obtained (figure 6.15).

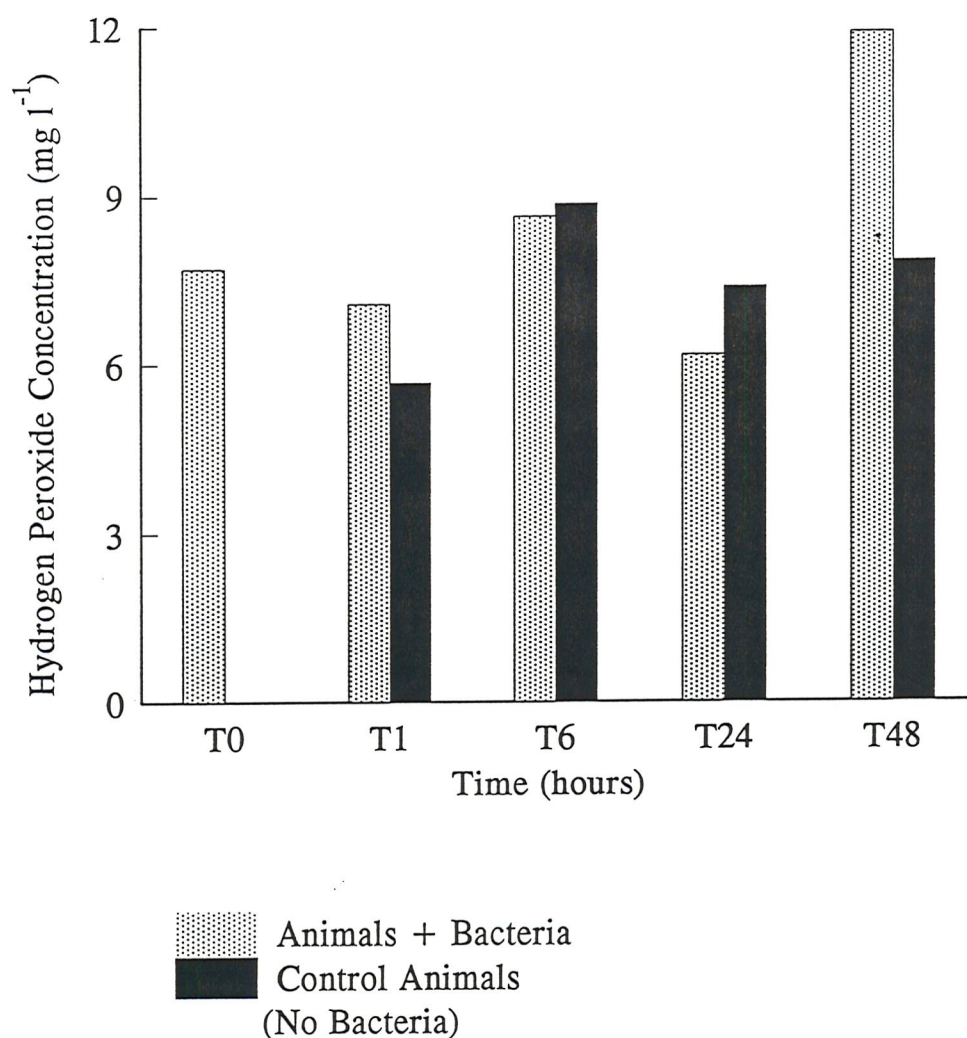


Figure 6.15 Mean values, from monthly sampling measurements, of hydrogen peroxide production (mg l⁻¹) in *Ostrea edulis*, following challenge with *Vibrio anguillarum*.

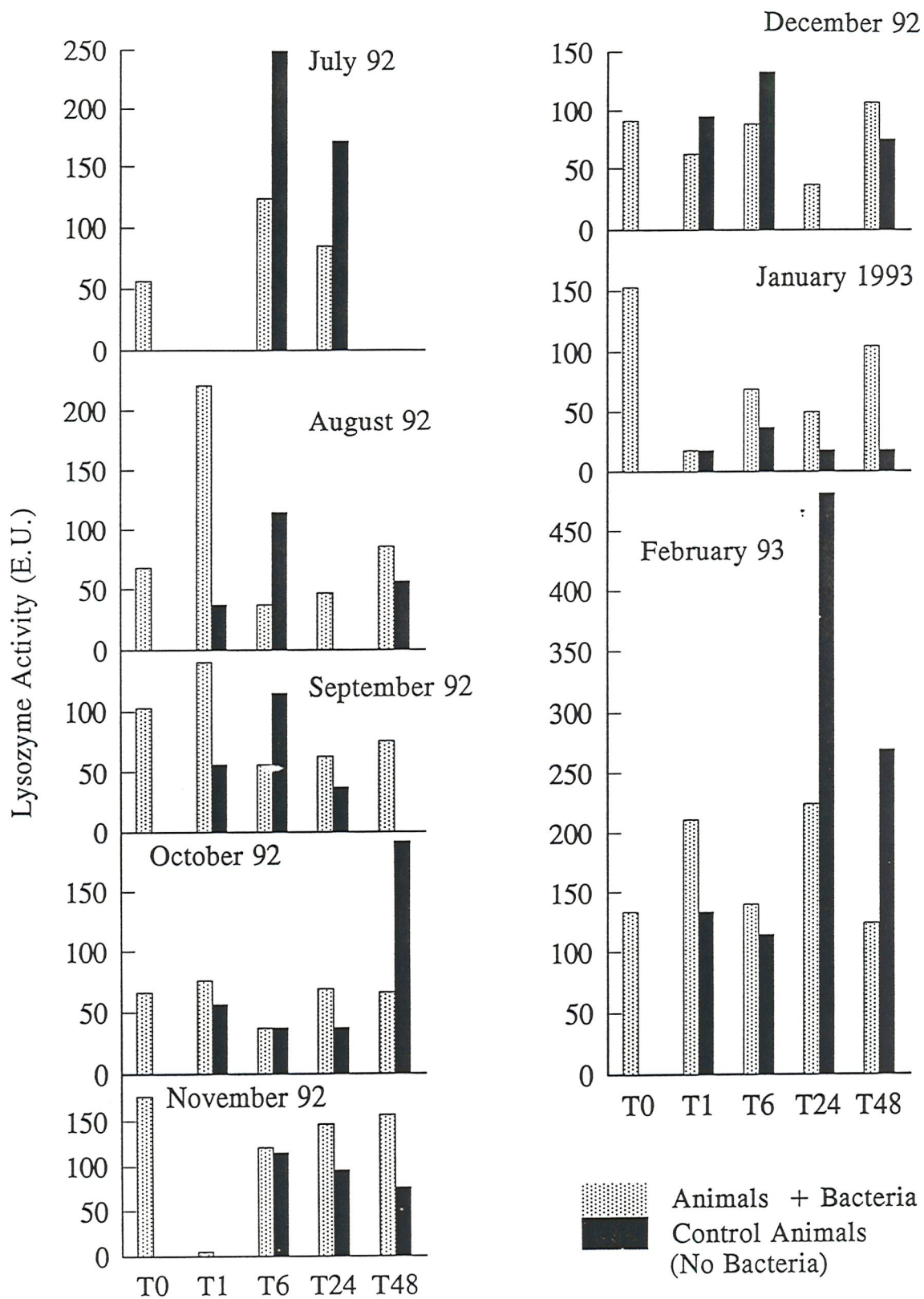


Figure 6.16 Monthly variation in haemolymph lysozyme activity (enzyme units), following challenge with *Vibrio anguillarum*, of *Ostrea edulis* taken from the Sowley sampling site. Means, $n = 5$ for T0, $n = 3$ for all other experimental challenges, $n = 1$ for all controls

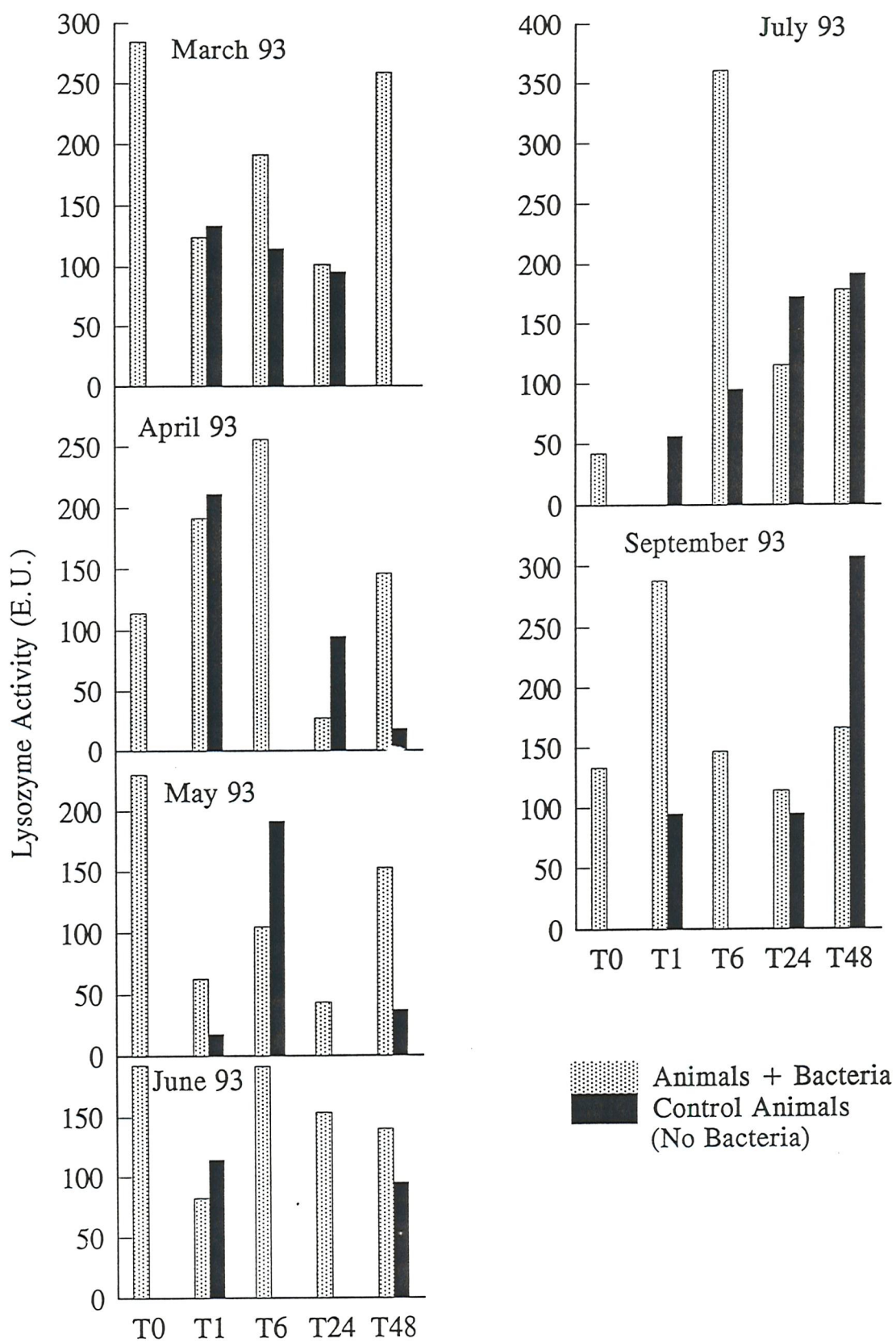


Figure 6.16 *continued.*

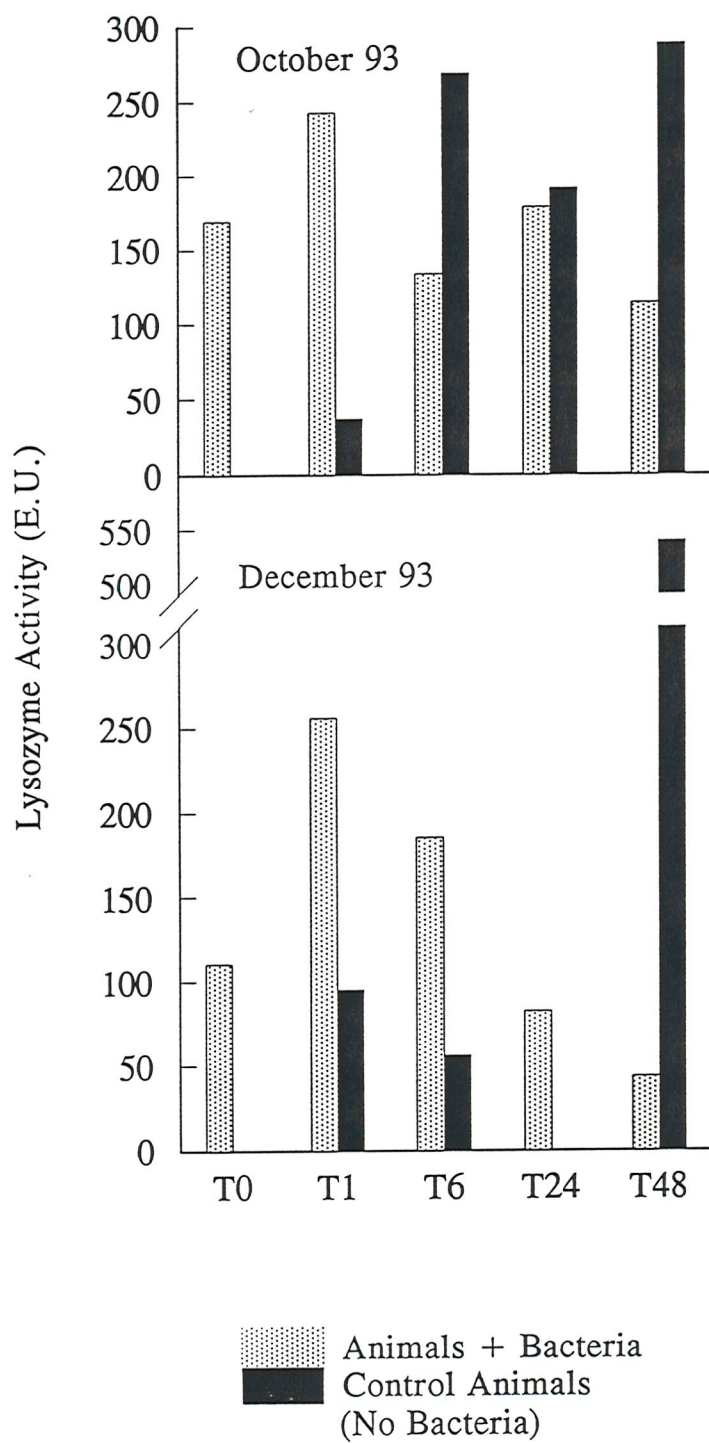


Figure 6.16 *continued.*

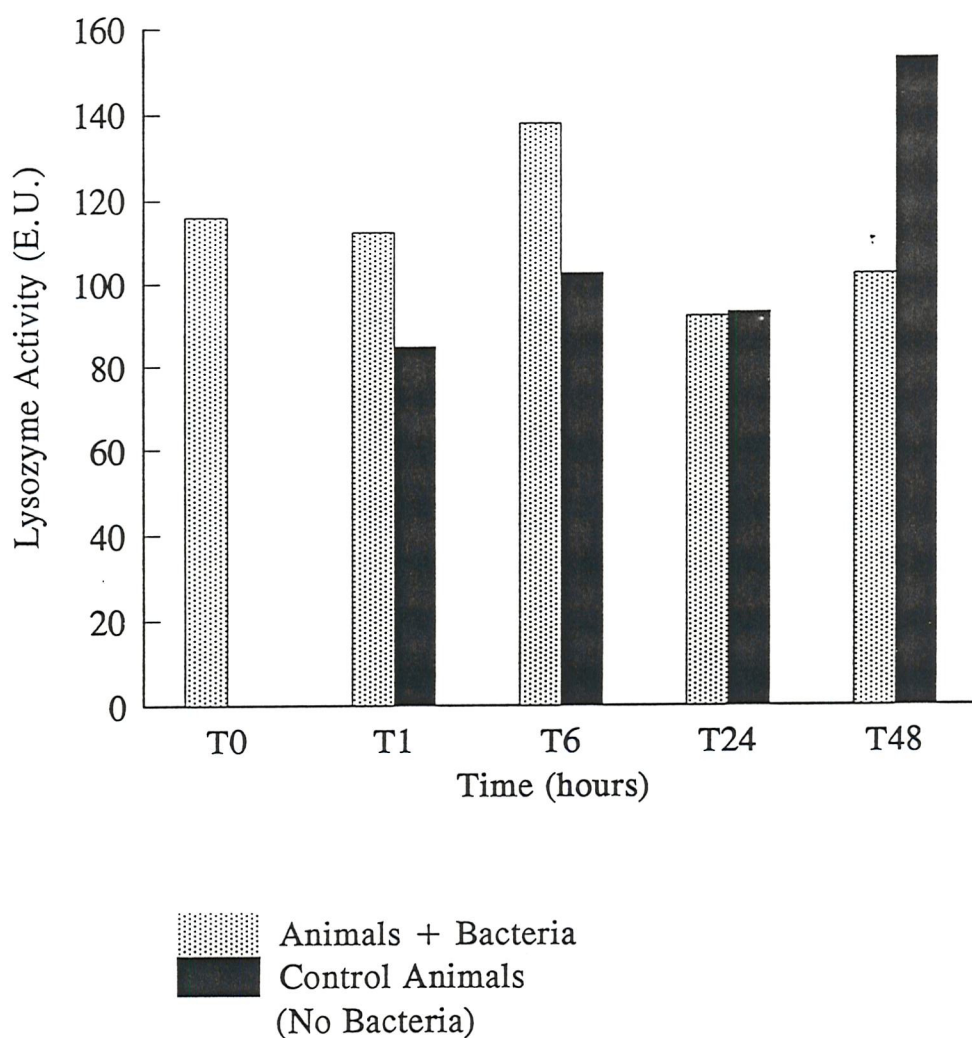


Figure 6.17 Mean values, from monthly sampling measurements, of lysozyme activity (enzyme units) in *Ostrea edulis*, following challenge with *Vibrio anguillarum*.

6.4 DISCUSSION

6.4.1 Environmental Parameters

The data presented in this study are comparable with the environmental variables measured in a two year programme at the Sowley site by Hawkins and Hutchinson (1990). The salinity and temperature measurements, in figures 6.03 and 6.04 respectively, have a highly significant correlation; the maximum salinity of 36.5‰ coincided with high summer temperatures, probably as a result of surface evaporation. The minimum salinity observed (32.2‰) was not as low as the 30.0‰ recorded by Hawkins and Hutchinson (1990) although both figures were recorded during the winter months.

The changes observed in temperature and salinity, and the total numbers and size of suspended particulates (figures 6.05 to 6.07) are highly influenced by river run-off, and in particular from the nearby Lymington and Beaulieu rivers. Particle size distribution in summer was dominated by a very fine fraction, which was reflected by the modal size of the particles (figure 6.07). The increase in numbers of the 3.18-8.00 μm fraction during October 1992, and again in January and February 1993, also coincided with increases in the organic load of the water column (figure 6.08). This is probably indicative of an increase in phytoplankton from an autumn bloom and, possibly, from associated bacterial blooms. The increase in organic material in January and February, which appears a long time after any autumn bloom and before any spring bloom would be expected, also occurred when sediment input from fluvial sources increased. It may well be that the suspended sediment load had bacterial flora associated with it, with bacteria adhered to the surface of particulate matter. Increased particulate numbers in winter (figure 6.06) may also have occurred as a result of turbulent conditions causing re-suspension of sediment and detrital material, from the flocculation of decaying material from blooms or aggregations of zooplankton faecal material.

The increase in winter of the larger size fraction of particulates is reflected by the increase in modal particle size during winter. Although bacterial and phytoplankton counts were not made, the patterns of variation seen in total particulate numbers (figure 6.06) reflected the combination of monthly changes in spring and summer primary production, and the increases in fluvial inputs during

periods of increased river flow. When particulate numbers increased but the modal size remained small, as for October to December 1993, it may have been a result of increased bacterial numbers following a phytoplankton bloom (Purdie and Antai, 1987; Hawkins and Hutchinson, 1990).

The changes observed in organic load correlated significantly with varying particulate numbers and with temperature, and as with the changing size spectrum of particulate matter is probably influenced by blooms of both phytoplankton and, later, of bacteria. This increase in particulate numbers mentioned, between October and December 1993, coincided with increasing organic load, which lends further circumstantial evidence to these particulates representing a bacterial bloom. Maximum organic loads occurred in October 1992 and January/February 1993, which may be attributed to autumnal fluvial input of allochthonous material (Hawkins and Hutchinson, 1990). The inorganic load (figure 6.09) did not appear to show any sort of pattern, and consequently did not correlate with any of the other factors. Increased levels in January 1993 corresponded to high organic levels, which may have been a consequence of bacterial association with sediment, as mentioned earlier. However, unlike the data presented by Hawkins and Hutchinson (1990), the inorganic load did not match the total particulate load (figure 6.10), showing that the mineral content of the suspended load was highly variable and not always the majority constituent.

6.4.2 Amoebocytic Uptake of *Vibrio anguillarum*

The pattern of bacterial uptake demonstrated by large granulocytes of *Ostrea edulis* (figure 6.11) does not correlate with any of the environmental parameters. Table 6.02 shows certain significant differences existed; the values for summer 1992 (June to September) were lower than for autumn/winter (October to February), and were also slightly lower than for the corresponding period for 1993. Unfortunately, the eighteen month sampling period was too short to determine whether the differences observed were a result of seasonal changes, or from some other influence. The lack of significant correlation with temperature may be considered surprising, as it has been shown that rate of locomotion

increases with increasing temperature (Fisher, 1988b; Hawkins and Hutchinson, 1990). If locomotion and spreading is increased with temperature, then the number of potential contacts with bacteria would be increased, and thus the degree of phagocytosis would be expected to increase. The increased levels of phagocytosis from November 1992 to January 1993 compared with summer months of 1992, and to a lesser extent of 1993, show the inverse of this hypothesis occurred. It is possible that the increased levels of phagocytosis are linked more to the increased particulate levels, especially if there is a bacterial bloom following decay of an autumn phytoplankton bloom.

The presence of bacteria has been shown to have significant effects promoting phagocytosis, and related immunological processes, in molluscan haemocytes. Injection of heat-killed *Bacillus megaterium* has been shown to engage phagocytosis of *Mercenaria mercenaria* haemocytes (Cheng, 1988), and the presence of foreign protein has been shown to act as a stimulus to the spreading of amoebocytes in the gastropod *Bulinus truncatus rohlfsi* (Cheng and Guida, 1980). Certainly there is chemotactic attraction of haemocytes by certain live bacteria, causing increased levels of phagocytosis, which is not demonstrated by the presence of heat-killed bacteria (Cheng and Howland, 1979). However, these workers also discovered that there was no chemotactic attraction of *Crassostrea virginica* haemocytes by live or heat-killed *Vibrio parahaemolyticus* bacteria. It is quite possible that the *V. anguillarum* used for this study also did not exhibit any chemoattractant, so it would not be expected to promote phagocytosis.

Certain environmental and toxicological factors have also been demonstrated to have an inhibitory effect on haemocytic function. Some heavy metals have been proved to inhibit phagocytosis of latex beads by *Crassostrea virginica* haemocytes (Cheng and Sullivan, 1984), and of *Escherichia coli* by granulocytes of the same species (Cheng, 1988). The decreased levels of phagocytosis in the summer months may well be a result of a combination of factors, including those described. Increased environmental temperature, together with a greater amount of energy input into gametogenesis and spawning, would decrease the resources available for immunological processes (see section 3.4.3). Bacterial diseases and oyster mortality often occur during the summer, when water

temperatures and nutrient levels are elevated, and oysters exhibit rapid growth and gonad maturation (Perdue, 1983; Beattie *et al.*, 1988). These extreme conditions may act as external stressors, decreasing the oyster's defence mechanism. The same relationship between environment, host and bacteria has been suggested in *Crassostrea gigas* reared in Japan (Tamate *et al.*, 1965). It is highly likely that a similar combination of factors has contributed to the reduced phagocytic capacity observed in the *Ostrea edulis* sampled from Sowley. Unfortunately, no quantitative records were made of the reproductive state of the oysters sampled, although it was noted that several animals opened during the summer months of both 1992 and 1993 were brooding larvae (Brooks, *pers. obs.*).

No correlation was found between the phagocytic index employed and the other immunological measures of hydrogen peroxide and lysozyme production. Moore and Gelder (1985) showed that *Mercenaria mercenaria* which demonstrated increased phagocytosis, also had increased haemolymph enzyme activity. It was expected that a similar phenomenon would occur in the present study, as contact between bacteria and haemocytes is required for both respiratory burst activity (producing hydrogen peroxide) and for phagocytosis. That no correlation was found may have resulted from the slightly different protocols used for each index, with phagocytosis being measured by incubation of a haemolymph sample directly with a bacterial sample, unlike the peroxide assay which incubated a whole animal with the bacteria. It would have been desirable to make haemocytic measurements of rates of locomotion, cell size change and bacterial uptake, over the same 48 hour time period of the other haemolymph assay, but time restrictions made this impractical.

6.4.3 Haemolymph Immunological Indices

Quantification of hydrogen peroxide production showed a rise in concentration during the winter months, with minimal activity usually occurring in summer (figure 6.12). Comparison with the environmental data showed no significant correlations with these variables, but this may well have been caused by the variability of peroxide measurements. The minimum concentrations of

hydrogen peroxide occurred at times when summer temperature was at its maximum. This may be indicative of the environmental and physiological stress effects described in the previous section; with energy being predominantly partitioned towards reproductive strategies, less is then available for immunological processes. As animals recover their energetic resources after spawning, bacterial blooms are occurring, following the phytoplankton blooms. This would lead to increased pathogenic loads within the oysters as this bloom is flushed out of Southampton Water. A resulting increase in peroxide concentration in response to this would not be observed until after the spring bloom, i.e. in summer, when the response appears to be inhibited by increased temperature and spawning stress, or the autumn bloom, i.e. the late autumn or early winter as shown in figure 6.12.

Similar patterns in hydrogen peroxide production were demonstrated in *Ostrea edulis* from the Solent by Hawkins and Hutchinson (1990). They were unable to correlate the fluctuations observed with environmental parameters, but a decline in May to July 1989 was coincident with the main brooding period for the Solent population. Oysters which were brooding larvae were found to have no detectable hydrogen peroxide in their haemolymph. The present study found minimum concentrations of hydrogen peroxide occurred in August and September 1992, similar to the nadir reached in August 1989 (Hawkins and Hutchinson, 1990). The increase from October 1992 (figure 6.12) occurred as temperature began to drop below 10°C (figure 6.04) - this temperature has been described as important in determining a switch in *Ostrea edulis* physiology from summer to winter states or *vice versa* (see section 3.4.4). Hydrogen peroxide levels continued to increase to a maximum in January 1993, and remained relatively high, before a decrease in April 1993 when temperatures began to increase above 10°C. Hawkins and Hutchinson (1990) also found changes in peroxide concentration occurred throughout transitions at this temperature and that temperature shocked animals tended to show a reduction in hydrogen peroxide concentration. Similar patterns appeared to occur in the present study.

The lysozyme results for monthly variation show no such patterns (figure 6.13) with no significant changes over time. The recorded activities exhibited large

variability, with some animals having no detectable activity, making it extremely unlikely that significant correlations would exist. However, there is a statistically significant, negative correlation between lysozyme and salinity. Although there was no significant relationship with temperature, it has been noted that summer mortalities occur as a result of changing environmental conditions. The lowest levels of lysozyme activity occurred during the summer months of 1992, and reached a 1993 nadir in August; correspondingly, the highest levels of activity occurred during winter, as the salinity fell to minimal levels. These results would suggest that the oysters are at their most susceptible to pathogenic infection in the warmer months, when salinity is high.

From the data presented it is not possible to determine whether decreased salinity in winter occurred as a result of increased rainfall or river run-off, but Powell *et al.* (1992) demonstrated that *Perkinsus marinus* infection in *Crassostrea virginica* was reduced as a result of low salinities. Since temperature and salinity have been shown to modify the oyster's immune system (Fisher and Newell, 1986; Fisher and Tamplin, 1988) this is not surprising, nor is it when the optimal conditions for *C. virginica* survival appear to be estuarine in nature (Galtsoff, 1964). It is therefore probable that the increase in lysozyme activity with decreasing salinity is coincidental, with little real significant effect. Optimal lysozyme production in *Ostrea edulis* was recorded at a salinity of 34‰ by Hawkins and Hutchinson (1990), so it would be expected that summer conditions would give the rise to maximum activities. As this was not the case, it is highly likely that a complicated series of interactions occurred, as the animal's physiological and metabolic processes, especially reproductive ones, and the environmental conditions acted to produce the variable results obtained.

Hawkins and Hutchinson (1990) observed a significant increase in lysozyme activity in June 1989, and to a lesser extent the following month. This coincided with a significant increase in bacterioplankton in the water, but no apparent source for the bacteria was found. Red tides caused by the phototrophic ciliate *Mesodinium rubrum* have been shown to result in high levels of associated *Vibrio* spp. (Romalde *et al.*, 1990), and as blooms of this ciliate were recorded in Southampton Water prior to the occurrence of the increase levels of lysozyme, this

was thought the most likely source of bacteria. These bacteria were flushed from the estuary, into the Solent, where their presence was suggested to cause the observed increase in haemolymph lysozyme concentration (Crawford *et al.*, 1993). *M. rubrum* was not thought to be the cause as it minimised flushing from the estuary by vertical migration, avoiding the surface layer during ebb tides (Crawford and Purdie, 1992). No *Mesodinium* blooms were recorded during the 1992-93 period of this investigation (Brooks, *pers. obs.*), and consequently there was no peak in lysozyme activity resulting from the lack of corresponding bacterial blooms. It is also unlikely that the higher levels of lysozyme observed in November 1992 and March 1993 were a result of particulate stimulation, as was recorded in a previous study (Hawkins and Hutchinson, 1990), since other months with high particulate loads did not demonstrate this increase.

6.4.4 Effects of Bacterial Challenge on Immunological Indices

Whilst monitoring the monthly changes in oyster immunology, it was also necessary to find out how pathogen challenge would effect animals under the changing environmental conditions. The response of hydrogen peroxide production to incubation with *Vibrio anguillarum*, over a 48 hour time period, is presented in figure 6.14. Initial examination suggested there were no patterns of any sort; two-way ANOVA showed there to be no significant differences between any of the months, probably as a result of the small sample size taken, and the inherent variability within the population (as indicated in the previous section). However, there was a significant increase in peroxide concentration after 48 hours incubation with bacteria, once the monthly effects were taken into account. The lack of change in activity during the first 24 hours, followed by this increase may be a consequence of one or more different factors. The respiratory burst activity which results in hydrogen peroxide production is very rapid (Bachere *et al.*, 1991) giving an immediate response to pathogenic invasion. It would be expected that as soon as a pathogen, in this case *Vibrio anguillarum*, is found within an animal, there would be a corresponding increase in microcidal agents. Since there was no increase until 48 hours post-inoculation, it must be assumed that this was the time

taken for the bacteria to invade the haemolymph, and possibly other tissues. The results for control animals showed no such changes in peroxide production, so the effects of incubating the animals in enclosed vessels may be discounted as a cause of peroxide increase. Unfortunately, the protocol employed to quantify this index did not measure the bacterial numbers before or after the inoculation, so the degree to which the *Vibrio* multiplied in numbers was not ascertained. It may have been that no response is elicited in the oysters until bacterial numbers have reached a certain threshold number, which may have taken 48 hours to reach. The effect of incubating bacteria *in vitro* with a haemolymph sample was not possible, as explained in section 6.2.4, since the broth interfered with the reagents used. It would also have been desirable to increase the sample size of oysters assayed, but the time constraints of the experiments did not make this possible. Modifications of the protocol to allow for these difficulties were made for the challenge of *Crassostrea gigas* under different conditions, and are described in the next chapter.

The results of lysozyme activity after challenge with *Vibrio anguillarum* were more variable than those for the hydrogen peroxide concentration (figures 6.16 and 6.17), and reflected the large variations demonstrated by the monthly sampling results. Two-way ANOVA showed no significant trends existed either monthly or over the 48 hour experimental period, and consequently no definite conclusions could be drawn from the data. If peroxide production is the more immediate defence mechanism, then it would be expected that lysozyme activity would increase soon afterwards. Lysozyme and related enzymes rely on degranulation of lysosomes found within granulocytes (Foley and Cheng, 1977; Cheng, 1986), and is increased in rapidly phagocytosing cells (Foley and Cheng, 1977). This requires contact with the pathogen which, from the increase in peroxide production, had probably occurred. If the time of bacterial challenge had been extended beyond the 48 hour period, then an increase in lysozyme would have been expected to be found. However, the quality of the water would also have been getting worse, with ammonia build up and bacterial numbers flourishing, so this was not investigated further. The peak in lysozyme activity found with increasing bacterial numbers following a *Mesodinium rubrum* bloom

(Crawford *et al.*, 1993), also suggests that this bacterial challenge should have caused a lysozyme response. Infection with heat-killed *Bacillus megaterium* has been shown to increase aminopeptidase, lysozyme and acid phosphatase (a lysosomal enzyme) activity in haemocytes of the gastropod *Biomphalaria glabrata*, with the latter enzyme to a maximum two hours post-injection (Cheng *et al.*, 1978; Cheng and Butler, 1979). This was demonstrated both *in vitro* and *in vivo*. Haemolymph of *Mercenaria mercenaria* and *Crassostrea virginica* has been shown to contain β -glucuronidase (responsible for the hydrolysis of acid mucopolysaccharides in bacterial cell walls) on exposure to heat-killed *B. megaterium* (Cheng, 1986).

The results from this 48 hour bacterial challenge experiment are not conclusive, and require slight modification of the protocol to extend the period of study, increasing the sample size, and possibly adjusting the way in which these animals are inoculated. Despite this, the rise in hydrogen peroxide concentration after 48 hours is probably a result of bacterial invasion of body tissues and haemolymph, and indicate hydrogen peroxide as a primary defence mechanism, at any time of the year.

6.4.5 Summary

The environmental data measured was similar to that recorded by a previous study in 1988-90 (Hawkins and Hutchinson, 1990). The salinity and particulate load of the sample site appeared to be affected largely by fluvial sources and turbulence in winter, and by biological processes in the summer and autumn. Measurements of particle size suggested that large amounts of suspended load may have consisted of phytodetrital and bacterial material, and it is possible that there was an association between the surface of the sediment and bacterial flora.

The phagocytosis index showed low levels of *Vibrio anguillarum* uptake in summer 1992, increasing in winter, and then decreasing in summer 1993, although not as low as the 1992 levels. This was explained as resulting from temperature stress linked with the physiological stress of reproduction, during the summer

months, and was supported by the observation of low activity in animals which were brooding larvae. The increase in activity observed in late autumn was probably a result of an autumnal phytoplankton, and associated bacterial, bloom, as animals had recovered from the stress of spawning. Although no correlation was found with the other indices of stress, a link may have been deduced by modification of the experimental protocol used.

Hydrogen peroxide production throughout the sampling period followed a similar pattern to that of bacterial phagocytosis, with low summer values giving way to higher winter concentrations. This was again ascribed to temperature and spawning stress, decreasing energetic resources for immunological purposes during the summer period. The differences may also have occurred as a result of a potential switch between winter and summer physiology at 10°C. The lysozyme measurements proved very variable, although there was a negative correlation with salinity. No peaks in lysozyme production were observed during the sampling period, as with the study of Hawkins and Hutchinson (1990), but no *Mesodinium rubrum* bloom was observed either.

Bacterial challenge of oysters with *Vibrio anguillarum* gave a significant increase in hydrogen peroxide concentration after 48 hours, but not with lysozyme. This may have been caused by increasing bacterial numbers or it may reflect the length of time taken for bacteria to infiltrate body tissues. Modification of the experimental protocol, to extend the period of challenge, and to make haemocytic measurements of amoebocytic rate of locomotion, cell size change and phagocytic uptake of bacteria, may elicit more information, although the time-consuming nature of these assays may preclude their use at the same time as the other measurements.

CHAPTER SEVEN

EFFECTS OF BACTERIAL CHALLENGE ON THE IMMUNOLOGY OF *Crassostrea gigas*

CHAPTER 7

EFFECTS OF BACTERIAL CHALLENGE ON THE IMMUNOLOGY OF *Crassostrea gigas*

7.1 INTRODUCTION

Part of the original premise of the present study was to provide baseline data for aspects of oyster physiology and immunology in order to determine what the 'normal' response of an animal was to a given set of conditions. Once the degree of apparent immunocompetence was established, investigations would be made into how this changed as a result of pathogenic challenge, thus defining the degree to which the synergistic effects of the pathogen and environmental variables affected the oyster.

Chapter 2 set out the baseline physiological responses of *Crassostrea gigas* across a set of temperature and salinity variables normally encountered in British waters. The corresponding baseline immunological responses to these conditions are documented by Hawkins *et al.* (1993b). Chapter 6 described how the immunology of *Ostrea edulis* from the Solent changed monthly, in response to natural environmental variability in temperature, salinity and particulate matter (including bacterial and phytoplankton concentrations). The aim of the current chapter was to establish how bacterial challenge with *Vibrio anguillarum* would affect the immunology of *C. gigas* under a combination of temperatures and salinities. This data would thus add to our understanding of how seasonal environmental change affects the host defence response.

Although many bacterial diseases of shellfish are secondary to traumatic lesions or other forms of stress (Elston, 1989), their impact on some populations of oysters has proved highly significant and commercially devastating. The presence of bacteria in association with hinge ligament erosion was first recorded

by Elston *et al.* (1982) in juveniles of *Crassostrea virginica*, *Ostrea edulis* and *Mercenaria mercenaria*. This disease was subsequently confirmed to occur in *Tapes philippinarum* and *C. gigas* (Elston, 1984). While the causative agent of the ligament damage was identified as a *Cryptophaga*-like bacterium, other bacteria were associated with surrounding tissues. Some of these bacteria were *Vibrio* spp., whose ability to ferment sucrose and degrade xanthine has been shown to correlate with pathogenicity in bivalve mollusc larvae (Jeffries, 1982).

Vibrios are ubiquitous and seasonal in nature, with vibriosis of hatchery reared bivalves reported in Europe, North America and Australia (Elston, 1989) causing severe mortalities and limiting production. Other bacterial diseases have been shown to have some seasonality in their occurrence, such as Pacific oyster nocardosis which results in summer mortalities (Beattie *et al.*, 1988; Elston, 1989). The precise environmental factors which promote the disease are unknown, but oysters cultured in cooler, well-flushed waters may never show any signs of the disease (Elston, 1989). Heavy mortalities have been suffered by juvenile *Crassostrea virginica* during the summer months, with age/size specific and suspended culture influences (Bricelj *et al.*, 1992), while other bivalves such as *Mercenaria mercenaria* and *Ostrea edulis* from the same environment did not show any mortality. Bricelj *et al.* (1992) found two potential causative agents, associated with bacteria found in the mantle lesions (although not consistently), and the occurrence at the same time as the mortalities of a *Gymnodinium sanguineum* bloom. Although this dinoflagellate is not known to be toxic, it is possible that there is a resulting associated bacterial bloom, similar to that described for *Mesodinium rubrum* in the previous chapter.

These examples of bacterial insult to populations of bivalves, together with the occasional occurrence of heavy mortalities in the shellfish, show that although not necessarily a primary infectious agent, the opportunistic nature of bacteria make them a very important influence in molluscan culture. The need to determine environmental influences on the immunology of bivalves is extremely important, as suggested by the observations of seasonal change in haemocyte activity and lysozyme concentration in *Crassostrea virginica* (Chu and LaPeyre, 1989; Fisher *et al.*, 1989; Hawkins *et al.*, 1993c) and of salinity influences on haemocyte

function (Fisher, 1988b; Chu and LaPeyre, 1989).

7.2 MATERIALS AND METHODS

7.2.1 Collection and Maintenance of Animals

Crassostrea gigas were supplied by the MAFF Fisheries Laboratory, Conwy, throughout the year when required. Seasonal field effects were eliminated by an acclimatisation period to the aquarium conditions, followed by careful acclimation to experimental conditions. Animals were all approximately the same size (15-30 g wet shell) to minimise size effects. The oysters were maintained in the Department of Oceanography aquarium, in flow-through tanks and constant aeration, at ambient conditions (13-19°C, 31-33‰). Animals were kept in these tanks for a minimum ten day period before use in experiments; they were fed daily with a mixed algal ration of *Tetraselmis suecica*, *Isochrysis galbana* and *Phaeodactylum tricornutum*. Acclimation to experimental conditions was performed slowly, over a 48 hour period, under micro-processor control. Animals were maintained under the new conditions for a minimum of one week and usually for more than two weeks. These animals were also fed with a mixed algal ration, supplied slowly to the tank (approximately 5-10 ml min⁻¹) by a peristaltic pump.

7.2.2 Pathogen Challenge

Vibrio anguillarum bacteria were used to provide a pathogen challenge, quantifiable by immunological indices. Bacteria used for the assays were in nutrient broth solution, but were maintained in both broth and on solid agar media. The methods for these are given in Appendix A3.

The method used to challenge the oysters with bacteria was as described in section 6.2.4(c), with the following modifications :

- 1) Measurements of hydrogen peroxide and lysozyme were made after 1,3 and 6 hours only - the use of more animals in each experimental group, compared with those for chapter 6, meant it was not feasible to include measurements at 24 and 48 hours within the time limits of the assay. The initial response of oyster immunological processes was being investigated and when these assays were begun, the

results from chapter 6 had not been analysed so it was unknown that responses had not been recorded until 48 hours post-inoculation.

- 2) Experimental and control groups were measured in two separate experiments. 6 animals were assayed for each of three time periods, i.e. 18 experimental oysters were measured, and 18 animals were assayed as a control group.
- 3) The length of time taken to assay each individual animal was such that the final animal in each group would be measured almost one additional hour after its time of inoculation. Consequently animals 4-6 in each time period (1,3 or 6 hours) were set up in Beatson jars and inoculated at T0+30 minutes.
- 4) The control groups were inoculated with 100 μ l of autoclaved broth solution which had not been inoculated with bacteria.
- 5) Excretion measurements were made to determine (i) whether the pathogen challenge had an effect, compared with the control group, on animal physiological processes, and (ii) whether there was any additional physiological stress imposed with the animals being kept for 6 hours in an enclosed environment. Consequently a blank vessel was setup, without an animal but with a bacterial broth or blank broth inoculation. Excretion measurements were made as these were the easiest physiological traits to quantify; baseline responses have already been reported in chapter 2, and the experimental setup lent itself to this assay.
- 6) Bacteria were supplied in a concentration of greater than 4.00×10^4 bacterial cells per ml. This were checked using the counting methods described in Appendix A4.

7.2.3 Immunological Measurements

Hydrogen peroxide concentration and lysozyme activity were measured according to the methods described in sections 6.2.4(a) and 6.2.4(b) respectively.

7.2.4 Excretion Measurements

Nitrogenous excretion was measured by the method of Solorzano (1969) as described in section 2.2.2.

7.2.5 Statistical Analysis of Results

The results of laboratory experiments were expressed as mean values \pm standard error, unless otherwise stated. The number of observations at each point was six, as specified above. Where appropriate data were analysed using an unpaired t-test (or a Mann-Whitney test as a non-parametric alternative) for comparing two conditions, or a standard ANOVA (or a Kruskal-Wallis test as a non-parametric alternative) for comparing more than two conditions. Two-way ANOVA was used to analyse temporal variation in monthly immunological measurements under pathogen challenge, over the six hour period.

7.3 RESULTS

7.3.1 Hydrogen Peroxide Production

The effects of bacterial challenge on production of hydrogen peroxide, at different temperatures and salinities, are presented in figures 7.01 and 7.02 respectively. There were no apparent trends of any sort; this was supported by one-way ANOVA analysis which indicated no significant differences between any comparisons ($P > 0.05$). Comparisons of peroxide concentration under all conditions at 1 hour, 3 hours and 6 hours post-inoculation were also all non-significant ($P > 0.05$). At 33‰, two-way ANOVA indicated that when the effects of time were removed - i.e. regardless of the length of time the samples were incubated for - there was a significant difference between 5°C & 25°C ($P < 0.05$) and 15°C & 25°C ($P < 0.05$); the difference between 5°C and 15°C was non-significant ($P > 0.05$). At 25‰ (figure 7.02b), hydrogen peroxide concentrations at 5°C were significantly higher than at 15°C ($P < 0.05$), irrespective of time, but other differences were non-significant ($P > 0.05$). No significant differences were observed at 16‰ ($P > 0.05$). Differences between salinities at individual temperatures (figure 7.01), when the effects of time were accounted for, were only shown at 15°C, with significant differences between 16‰ & 25‰, and 25‰ & 33‰ ($P < 0.05$).

The measurements for the control animals under these conditions are presented in figures 7.03 and 7.04. These animals also showed no obvious trends, and had no significant differences between comparisons of different conditions ($P > 0.05$). Two-way ANOVA showed that the only significant effects which occurred were at 5°C and at 33‰. At the lowest temperature, when the effects of differences in salinity were accounted for, peroxide concentration decreased significantly between 3 and 6 hours ($P < 0.05$); other differences were non-significant ($P > 0.05$). At 33‰ salinity, when the effects of temperature were removed, there were statistically significant differences between 1 & 6 hours, and 3 & 6 hours post-inoculation ($P < 0.05$).

Comparisons of the experimental and control animals with the recorded baseline responses of hydrogen peroxide production under the same conditions, are indicated in table 7.01. All the significant differences occurred at low temperature

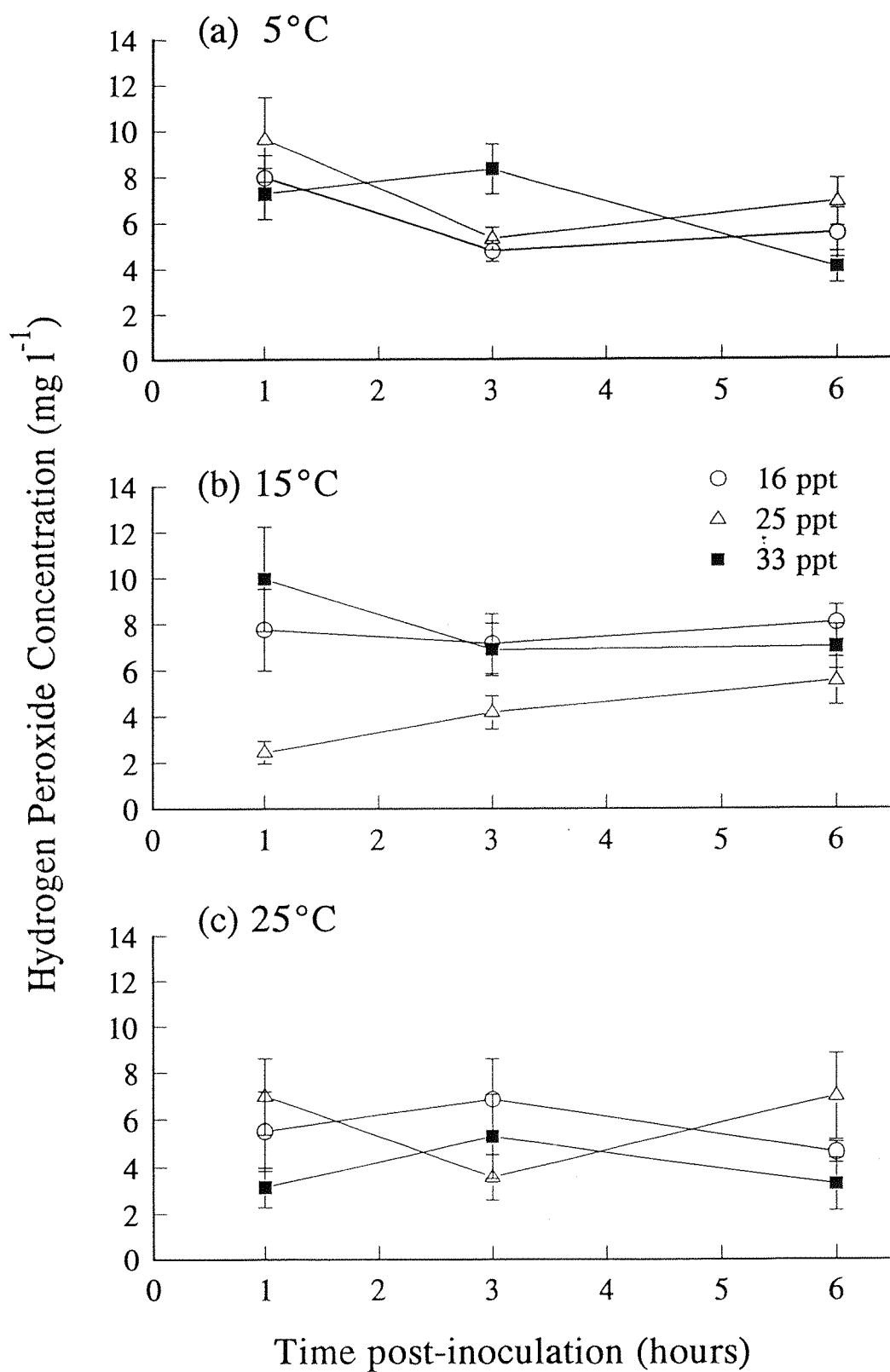


Figure 7.01 *Vibrio anguillarum* challenged *Crassostrea gigas*. Effect of salinity on hydrogen peroxide production (mg l⁻¹) at three different temperatures. Mean \pm SE, n = 6 at each point.

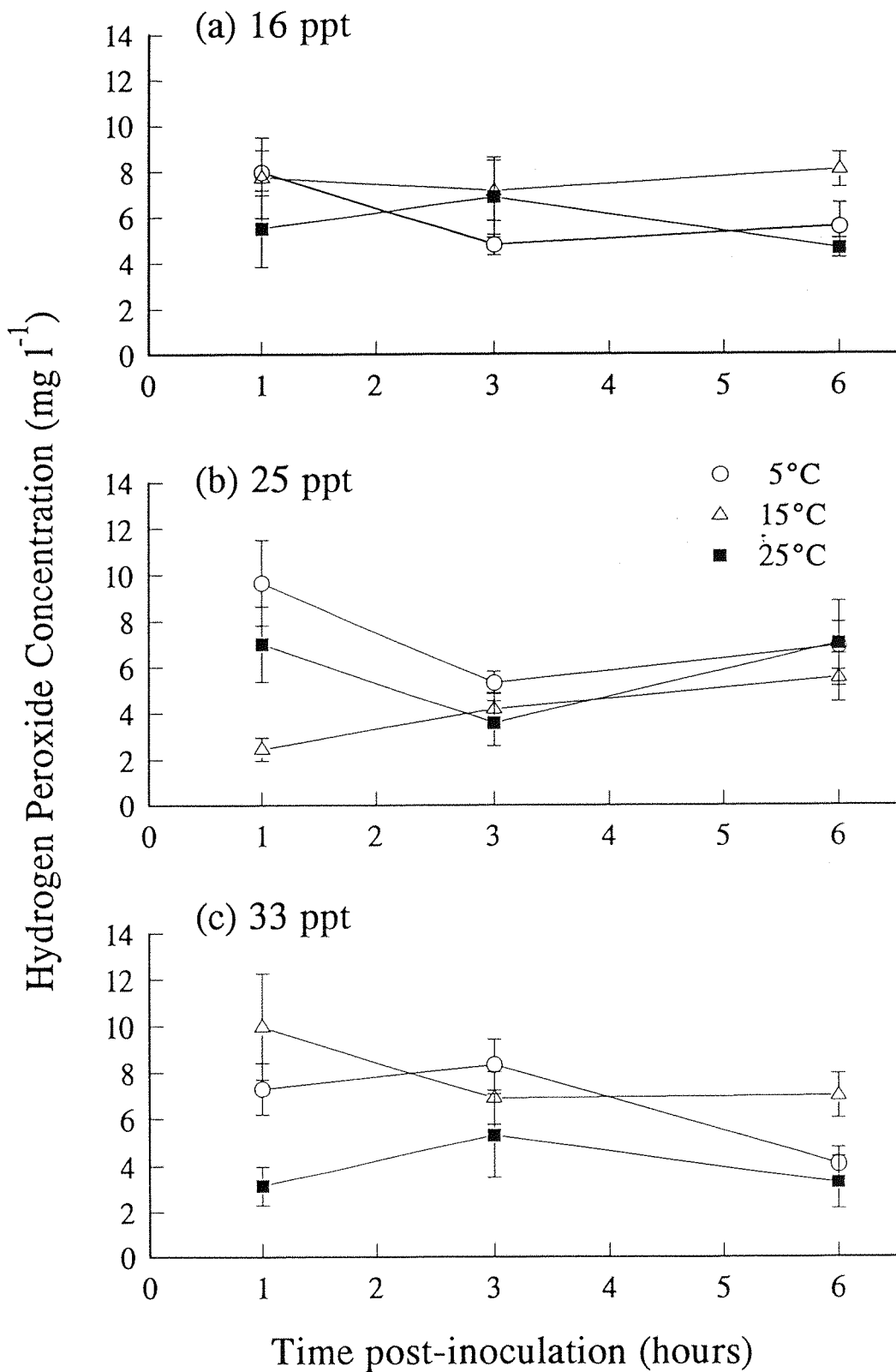


Figure 7.02 *Vibrio anguillarum* challenged *Crassostrea gigas*. Effect of temperature on hydrogen peroxide production (mg l⁻¹) at three different salinities. Mean \pm SE, n = 6 at each point.

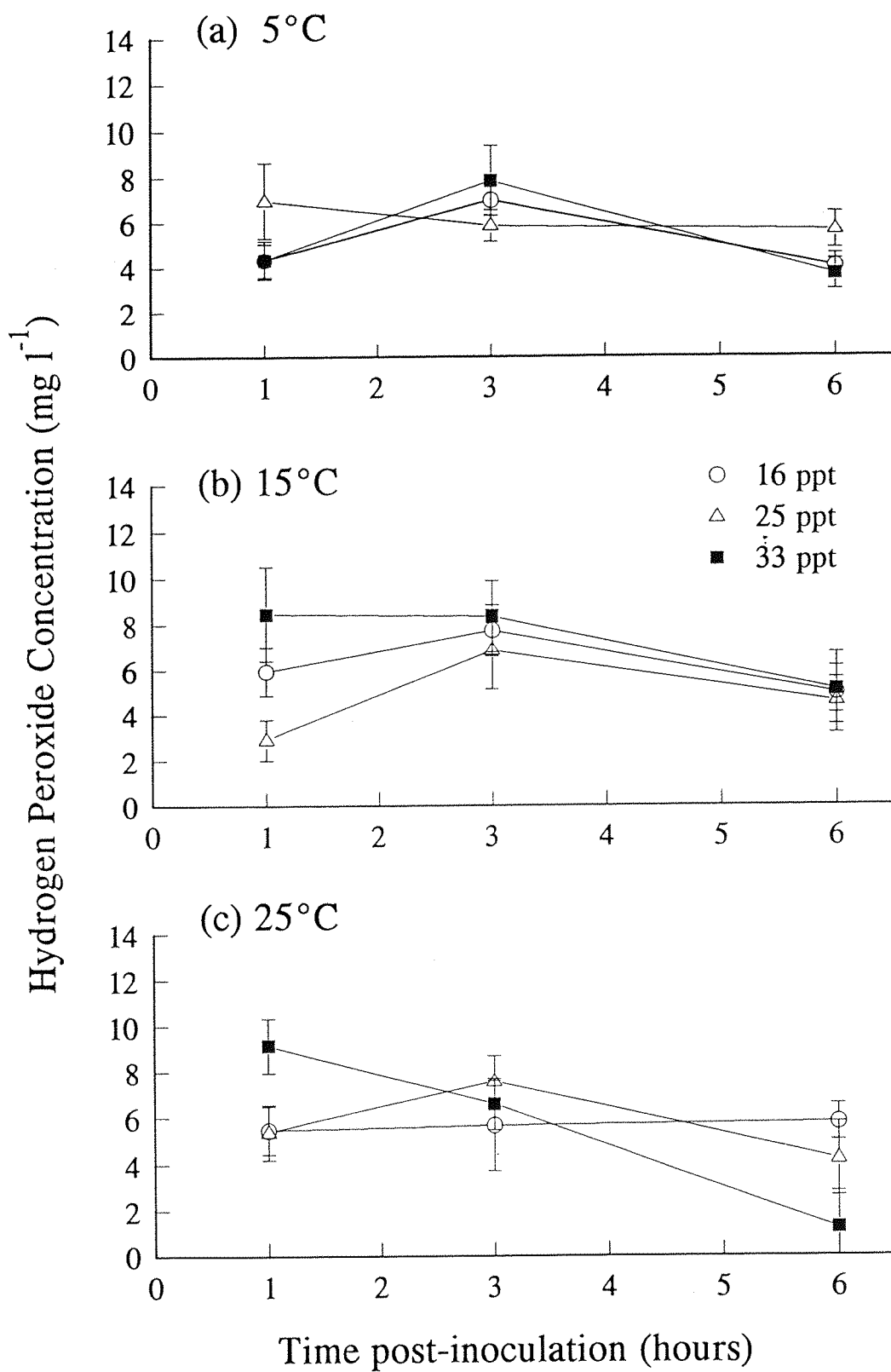


Figure 7.03 Control *Crassostrea gigas* (no bacteria). Effect of salinity on hydrogen peroxide production (mg l⁻¹) at three different temperatures. Mean \pm SE, n = 6 at each point.

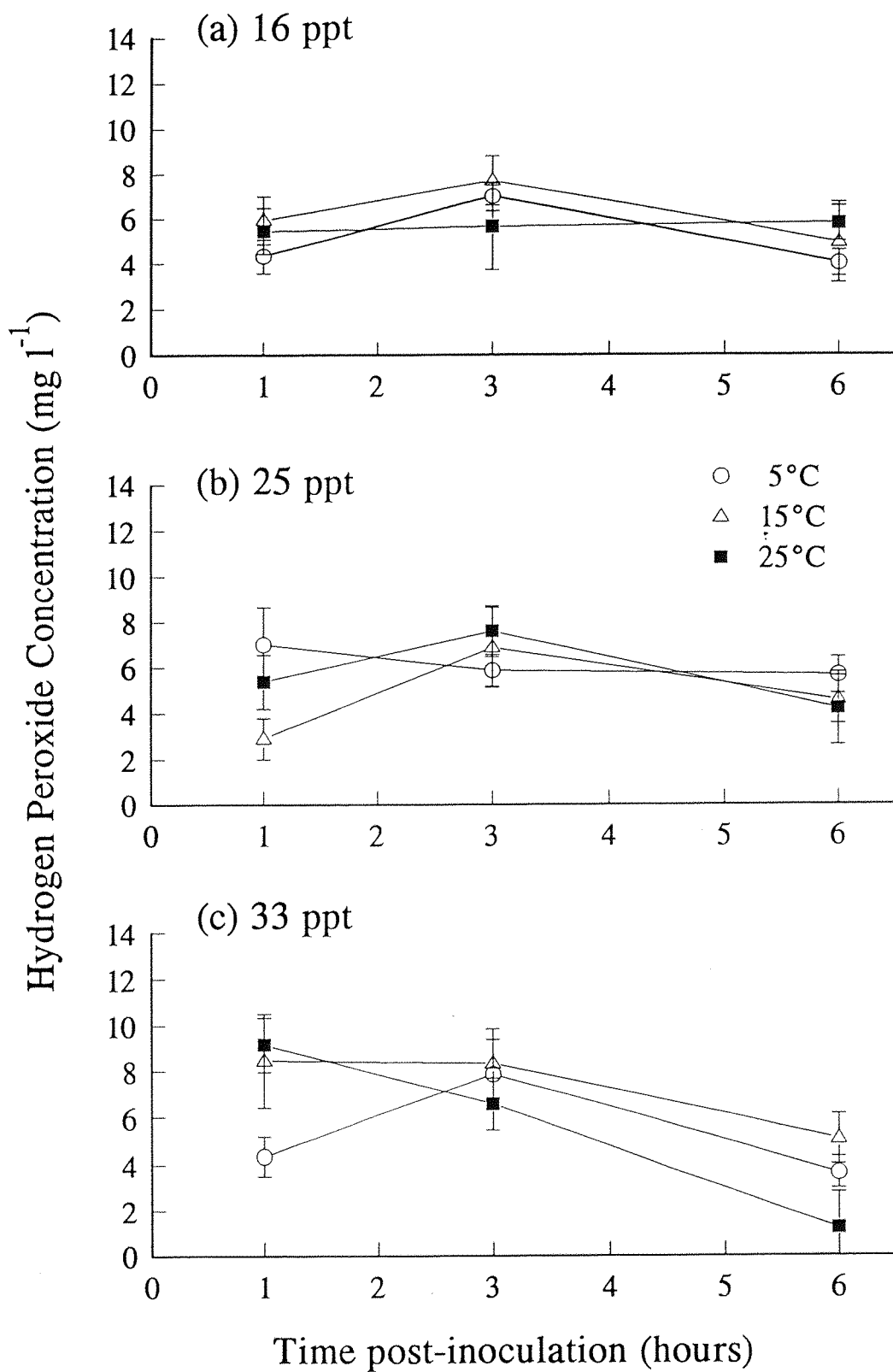


Figure 7.04 Control *Crassostrea gigas* (no bacteria). Effect of temperature on hydrogen peroxide production (mg l⁻¹) at three different salinities. Mean \pm SE, n = 6 at each point.

Temp./Sal.	Time	Experimental	Control
5°C/16‰	1	$P < 0.05$ (h)	n.s.
	3	n.s.	$P < 0.05$ (h)
	6	n.s.	n.s.
5°C/25‰	1	$P < 0.05$ (h)	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
5°C/33‰	1	$P < 0.05$ (h)	n.s.
	3	$P < 0.05$ (h)	$P < 0.05$ (h)
	6	n.s.	n.s.
15°C/16‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
15°C/25‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
15°C/33‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
25°C/16‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
25°C/25‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
25°C/33‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.

Table 7.01 Comparison of experimental and control results for hydrogen peroxide production with baseline responses. (Baseline data were obtained from Hawkins *et al.*, 1993b). Significant comparisons are shaded. n.s. = not significant ($P > 0.05$). (h) = higher than baseline, (l) = lower than baseline.

($P < 0.05$), and all the results were higher than the baseline responses.

7.3.2 Lysozyme Activity

The effects of *Vibrio anguillarum* challenge on lysozyme activity in *Crassostrea gigas*, under different temperature and salinity conditions, are presented in figures 7.05 and 7.06 respectively. There was no clear pattern of activity under the different conditions, but many animals demonstrated an increase between 1 and 3 hours after inoculation, followed by a decrease at 6 hours post-inoculation. However, the results were very variable. One-way ANOVA showed there were no statistically significant differences between any combination of comparisons ($P > 0.05$). Analysis of differences across all conditions after each of 1, 3 and 6 hours post-inoculation also demonstrated no significant differences ($P > 0.05$).

Two-way ANOVA indicated that at 5°C, when salinity effects were accounted for, there was a significant difference between lysozyme activity at 3 and 6 hours post-inoculation ($P < 0.05$). Other comparisons were not significantly different ($P > 0.05$). Significant differences were also recorded at 33‰, after the effects of temperature were allowed for. These occurred between 1 & 6 hours, and 3 & 6 hours post-inoculation ($P < 0.05$). At this salinity the effect of temperature depended on how long after inoculation the lysozyme activity was measured, although the only significant effect recorded was between 3 and 6 hours at 5°C ($P < 0.05$). All other comparisons were non-significant ($P > 0.05$).

One-way ANOVA analysis of the control results (figures 7.07 and 7.08) showed no significant differences occurred ($P > 0.05$). When salinity, temperature and time variables were accounted for by two-way ANOVA, the assays were still found to have no statistically significant differences between conditions ($P > 0.05$). When the experimental and control results of lysozyme activity were compared with baseline responses of *C. gigas*, the only significant differences to occur were at high temperature and high salinity ($P < 0.05$); the recorded challenge results were lower than the normal baseline responses measured.

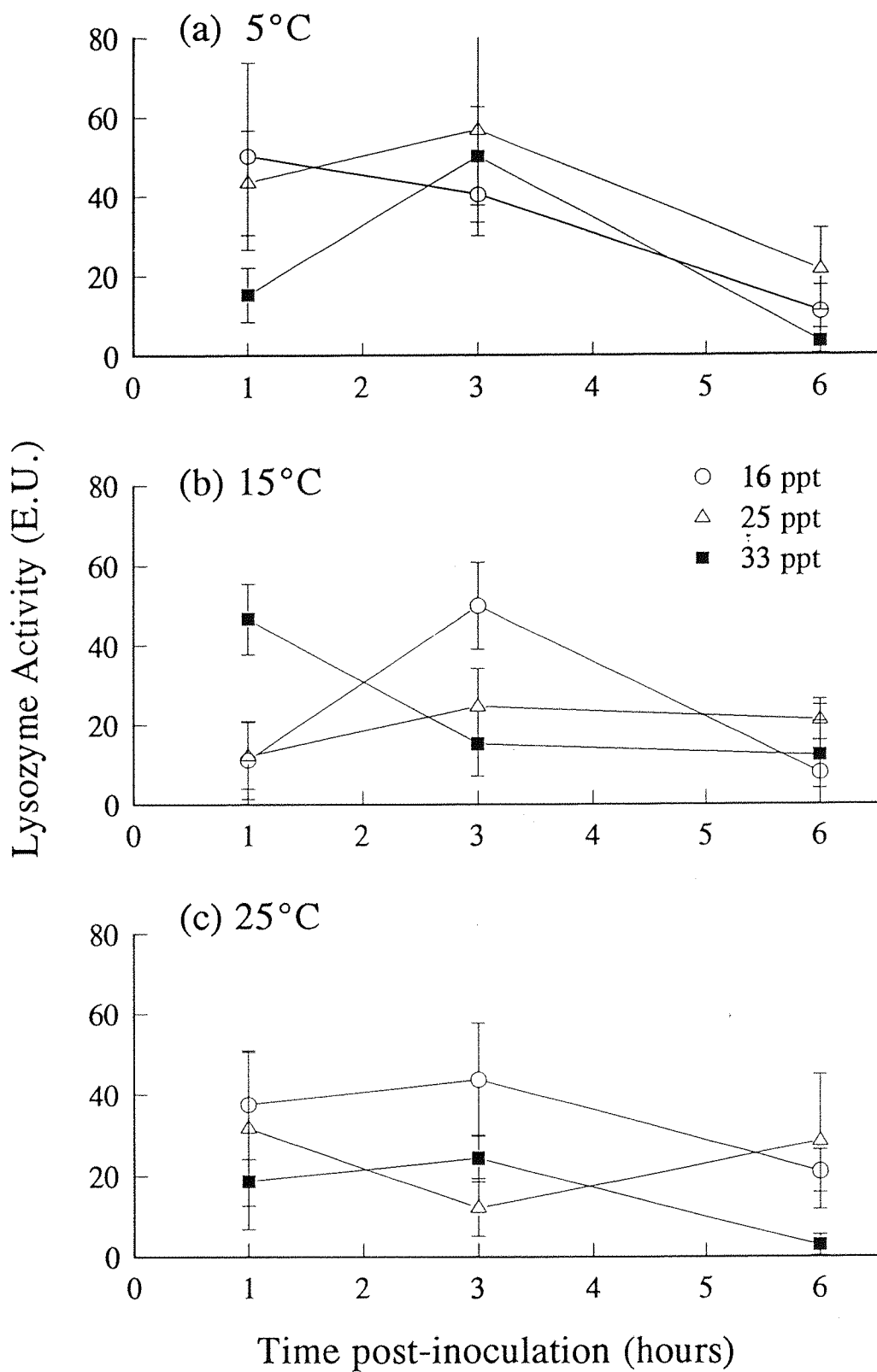


Figure 7.05 *Vibrio anguillarum* challenged *Crassostrea gigas*. Effect of salinity on lysozyme activity (enzyme units) at three different temperatures. Mean \pm SE, $n = 6$ at each point.

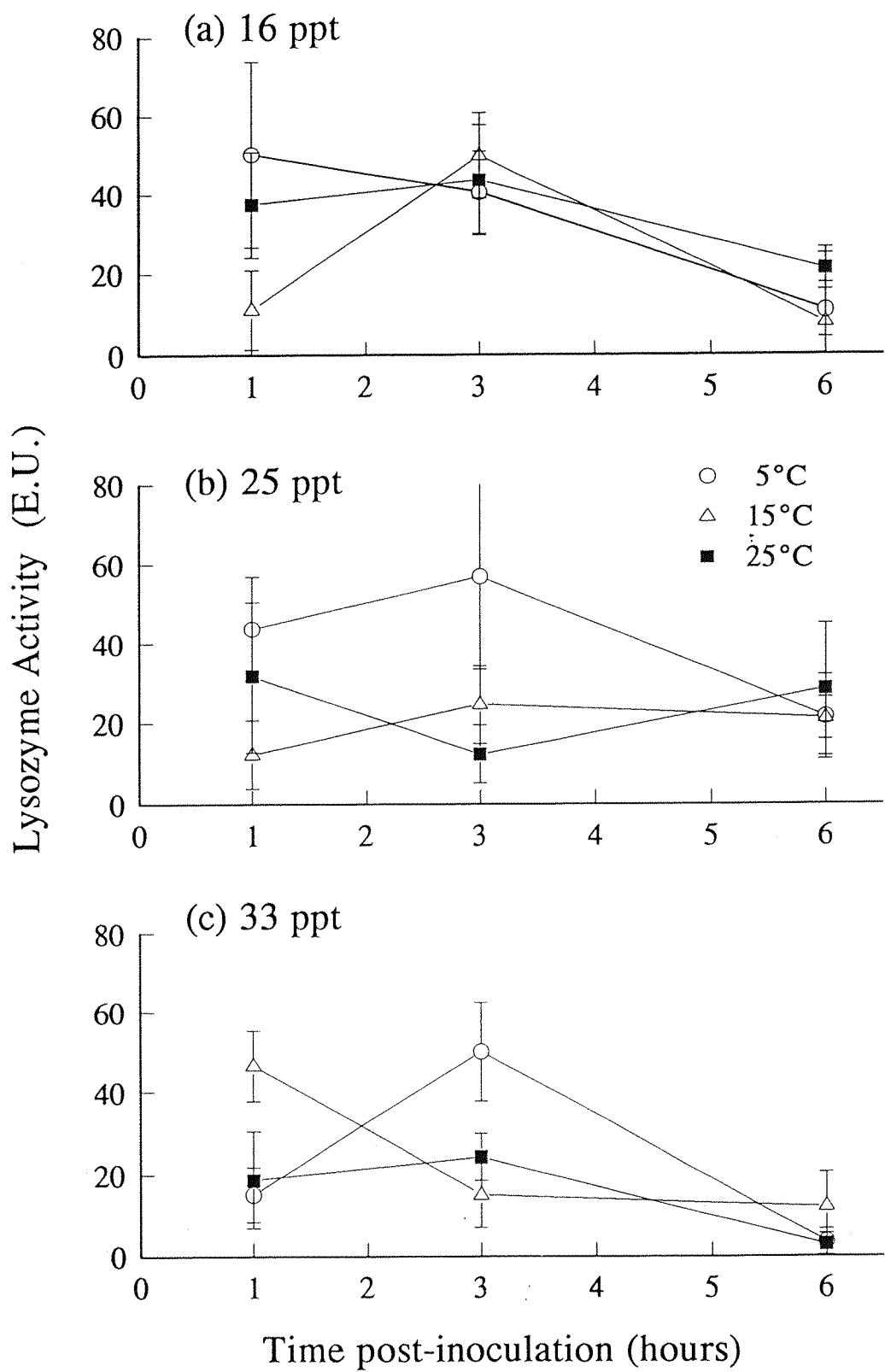


Figure 7.06 *Vibrio anguillarum* challenged *Crassostrea gigas*. Effect of temperature on lysozyme activity (enzyme units) at three different salinities. Mean \pm SE, $n = 6$ at each point.

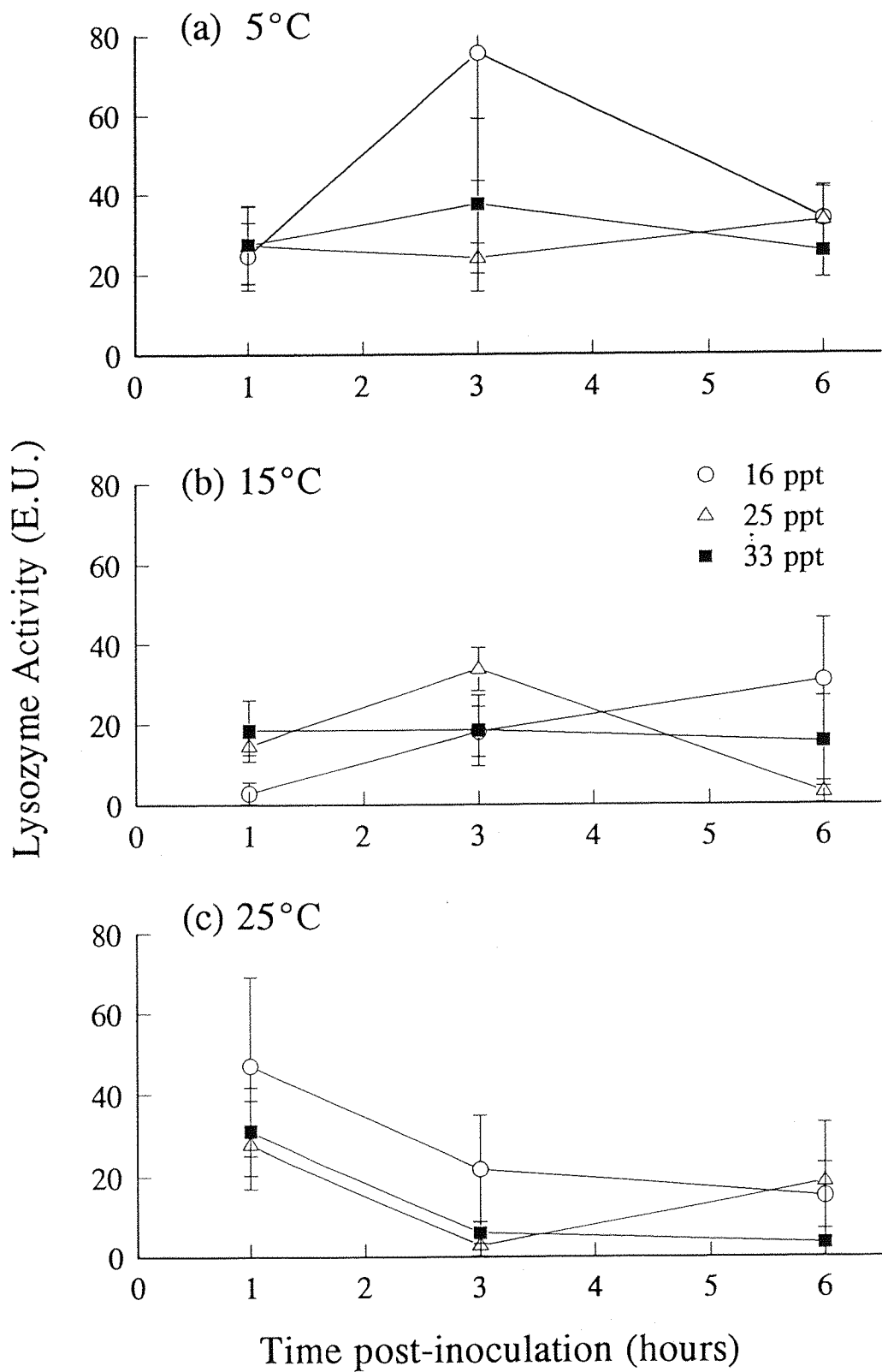


Figure 7.07 Control *Crassostrea gigas* (no bacteria). Effect of salinity on lysozyme activity (enzyme units) at three different temperatures. Mean \pm SE, $n = 6$ at each point.

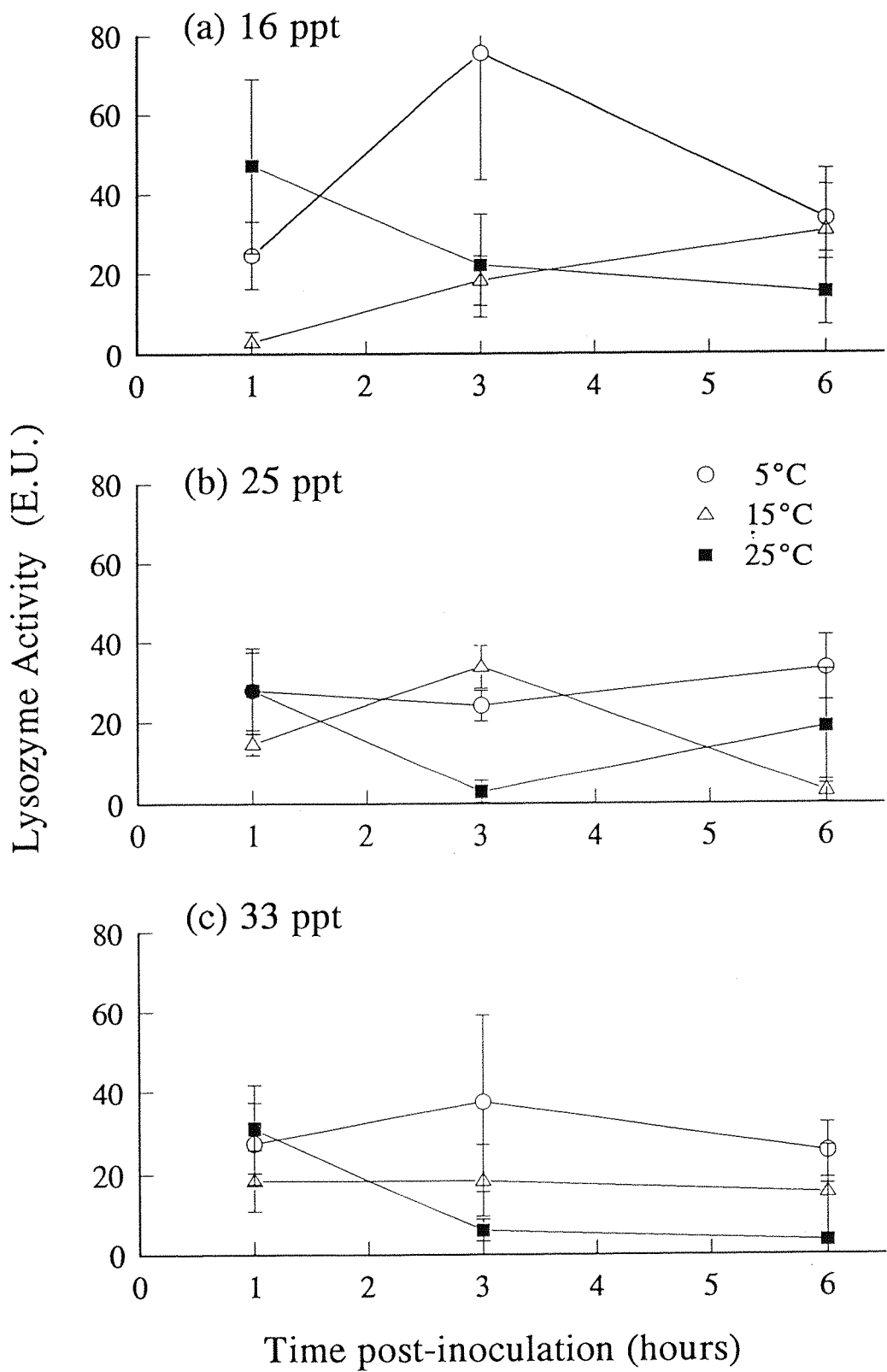


Figure 7.08 Control *Crassostrea gigas* (no bacteria). Effect of temperature on lysozyme activity (enzyme units) at three different salinities. Mean \pm SE, $n = 6$ at each point.

Temp./Sal.	Time	Experimental	Control
5°C/16‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
5°C/25‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
5°C/33‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
15°C/16‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
15°C/25‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
15°C/33‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
25°C/16‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
25°C/25‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
25°C/33‰	1	n.s.	n.s.
	3	n.s.	$P < 0.05$ (l)
	6	$P < 0.05$ (l)	$P < 0.05$ (l)

Table 7.02 Comparison of experimental and control results for lysozyme activity with baseline responses (Baseline data were obtained from Hawkins *et al.*, 1993b). Significant comparisons are shaded. n.s. = not significant ($P > 0.05$). (h) = higher than baseline, (l) = lower than baseline.

7.3.3 Nitrogen Excretion Rate

The results of *Vibrio* challenge on excretion rate in *C. gigas* at different temperatures and salinities are shown in figures 7.09 and 7.10. These results were generally less variable than the immunological measurements, with the majority of results showing a decrease in excretion rate with time. Statistically significant variation was recorded at the low temperature/salinity combinations, but the nature of these differences varied between conditions. At 5°C/16‰, there were significant differences between 1 & 3 hours, and 1 & 6 hours post-inoculation ($P < 0.05$); 5°C/25‰ showed a statistically significant difference between the experimental and control rates 1 hour after inoculation ($P < 0.05$). As the temperature increased to 15°C and 25°C there were no significant differences between combinations of conditions ($P > 0.05$). Comparisons of excretion rates 1 hour after inoculation showed that many of the conditions differed significantly - these differences are indicated in table 7.03. The excretion rates at 5°C/16‰ and 15°C/33‰ were significantly higher than at all other conditions ($P < 0.05$). Excretion rates did not differ significantly when compared at 3 hours post-inoculation ($P > 0.05$), and at 6 hours post-inoculation the only difference of significant interest occurred between 15°C and 25°C at 25‰ ($P < 0.05$).

Two-way ANOVA showed that many conditions demonstrated significant differences. At 33‰, significant differences were found between 5°C & 15°C, and between 15°C & 25°C, after allowing for time differences ($P < 0.05$); if these temperature differences were accounted for, excretion rate differences were also found between 1 & 3 hours, and 1 & 6 hours post-inoculation ($P < 0.05$). This meant many comparisons between combinations of temperature and time at 33‰ were also significantly different. Temperature differences, without time effects, were also found at 25‰, between 5°C & 25°C, and 15°C & 25°C ($P < 0.05$), but at low salinity those differences in excretion rate which were significantly different depended on which combinations of temperature and time were measured. Differences were also found at high and low temperature, when salinity and time effects were taken into account. A temperature of 25°C showed there to be significant differences between 16‰ & 33‰, and 25‰ & 33‰ ($P < 0.05$). At 5°C there were significant differences between 1 & 3, and 1 & 6

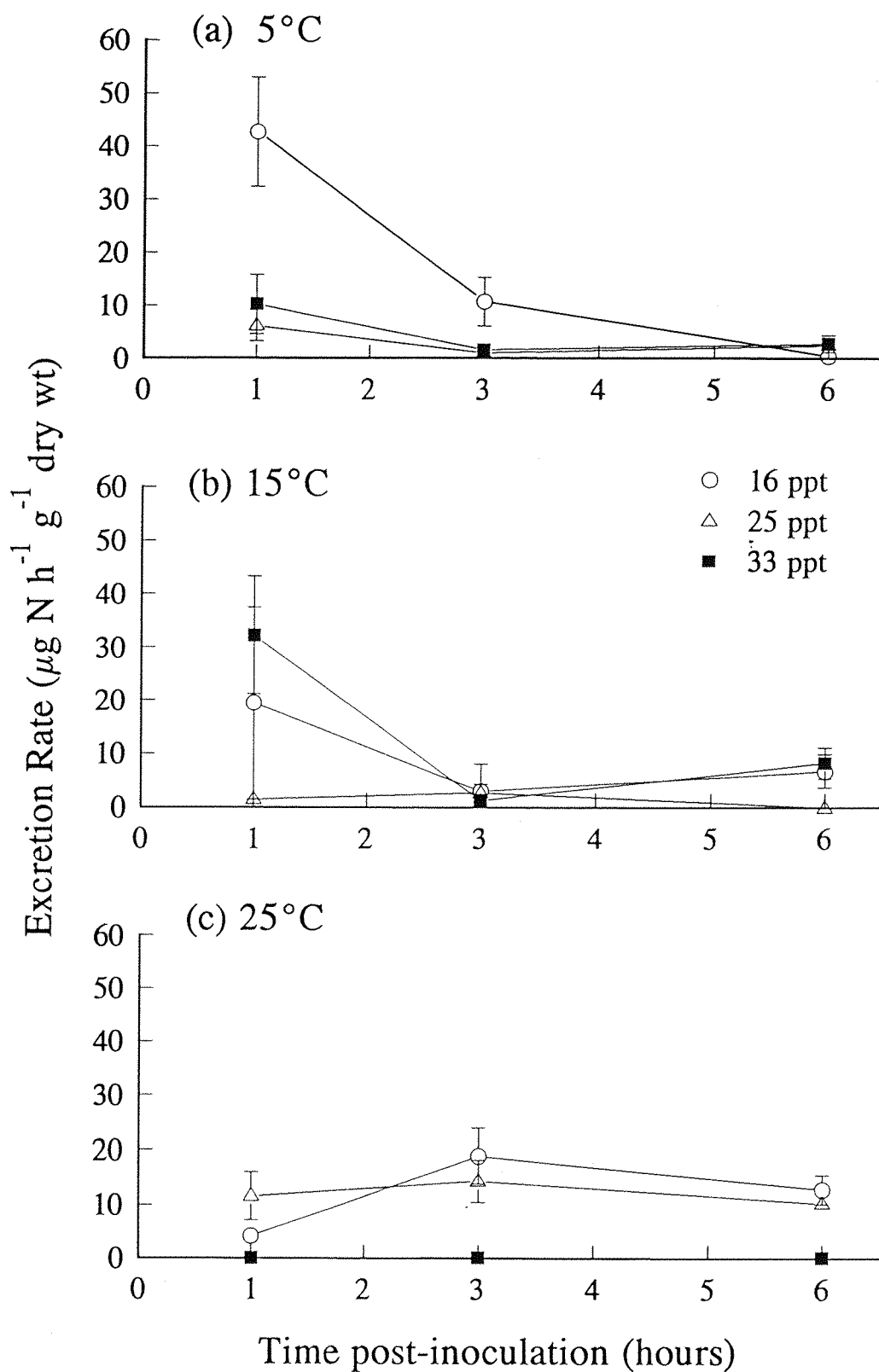


Figure 7.09 *Vibrio anguillarum* challenged *Crassostrea gigas*. Effect of salinity on excretion rate ($\mu\text{g N h}^{-1} \text{g}^{-1} \text{dry weight}$) at three different temperatures. Mean \pm SE, n = 6 at each point.

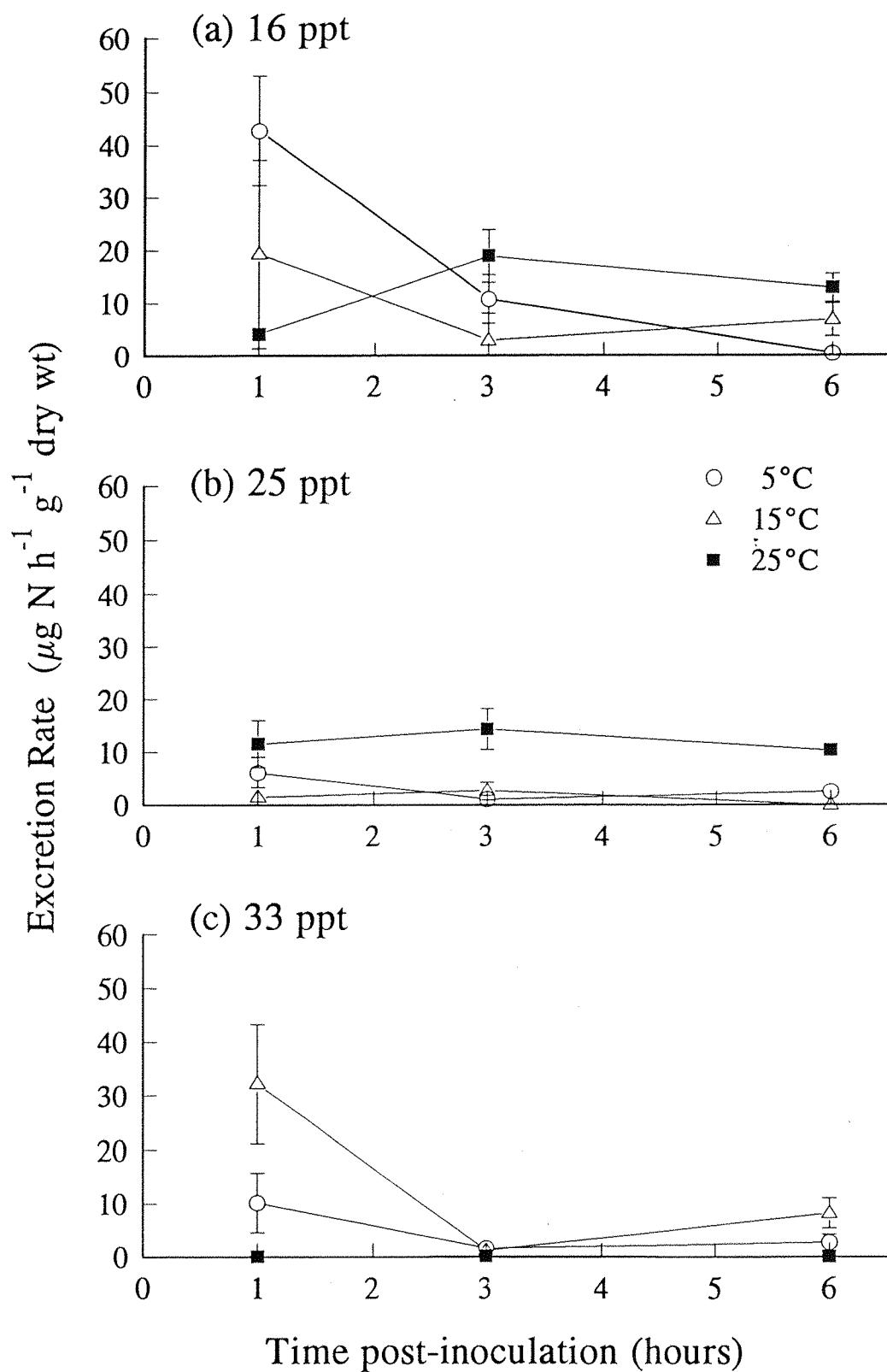


Figure 7.10 *Vibrio anguillarum* challenged *Crassostrea gigas*. Effect of temperature on excretion rate ($\mu\text{g N h}^{-1} \text{g}^{-1} \text{dry weight}$) at three different salinities. Mean \pm SE, $n = 6$ at each point.

	5°C/25‰	5°C/33‰	15°C/16‰	15°C/25‰	15°C/33‰	25°C/16‰	25°C/25‰	25°C/33‰
5°C/16‰	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$
5°C/25‰		n.s.	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	n.s.
5°C/33‰			n.s.	n.s.	$P < 0.05$	n.s.	n.s.	n.s.
15°C/16‰				n.s.	$P < 0.05$	n.s.	n.s.	n.s.
15°C/25‰					$P < 0.05$	n.s.	n.s.	n.s.
15°C/33‰						$P < 0.05$	$P < 0.05$	$P < 0.05$
25°C/16‰							n.s.	n.s.
25°C/25‰								n.s.

Table 7.03 Comparison of differences in excretion rates for experimental animals 1-hour post-inoculation with *Vibrio anguillarum*. Significant differences are shaded. n.s. = not significant ($P > 0.05$).

hours post-inoculation ($P < 0.05$), and also between 16‰ & 25‰, and 16‰ & 33‰ ($P < 0.05$), when temporal variability was accounted for.

The results of excretion rates in control animals under different temperature and salinity conditions are shown in figures 7.11 and 7.12 respectively. No significant differences were found by one-way ANOVA comparisons of combinations of conditions ($P < 0.05$). Two-way ANOVA analysis found no statistically significant differences at 25°C or 15°C, when salinity and time effects were allowed for, and at 5°C the only significant effect was found between 25‰ and 33‰, when time effects were removed ($P < 0.05$). Some temperature effects were found, when time effects were accounted for. These were between 5°C & 25°C, and 15°C & 25°C at both 25‰ and 33‰ ($P < 0.05$). All other comparisons were non-significant ($P > 0.05$).

The results of comparison of excretion rates in the control and experimental animals, with those rates measured as baseline responses (chapter 2) are presented in table 7.04. All the significantly different challenge responses were lower than the normal baseline excretion rates ($P < 0.05$).

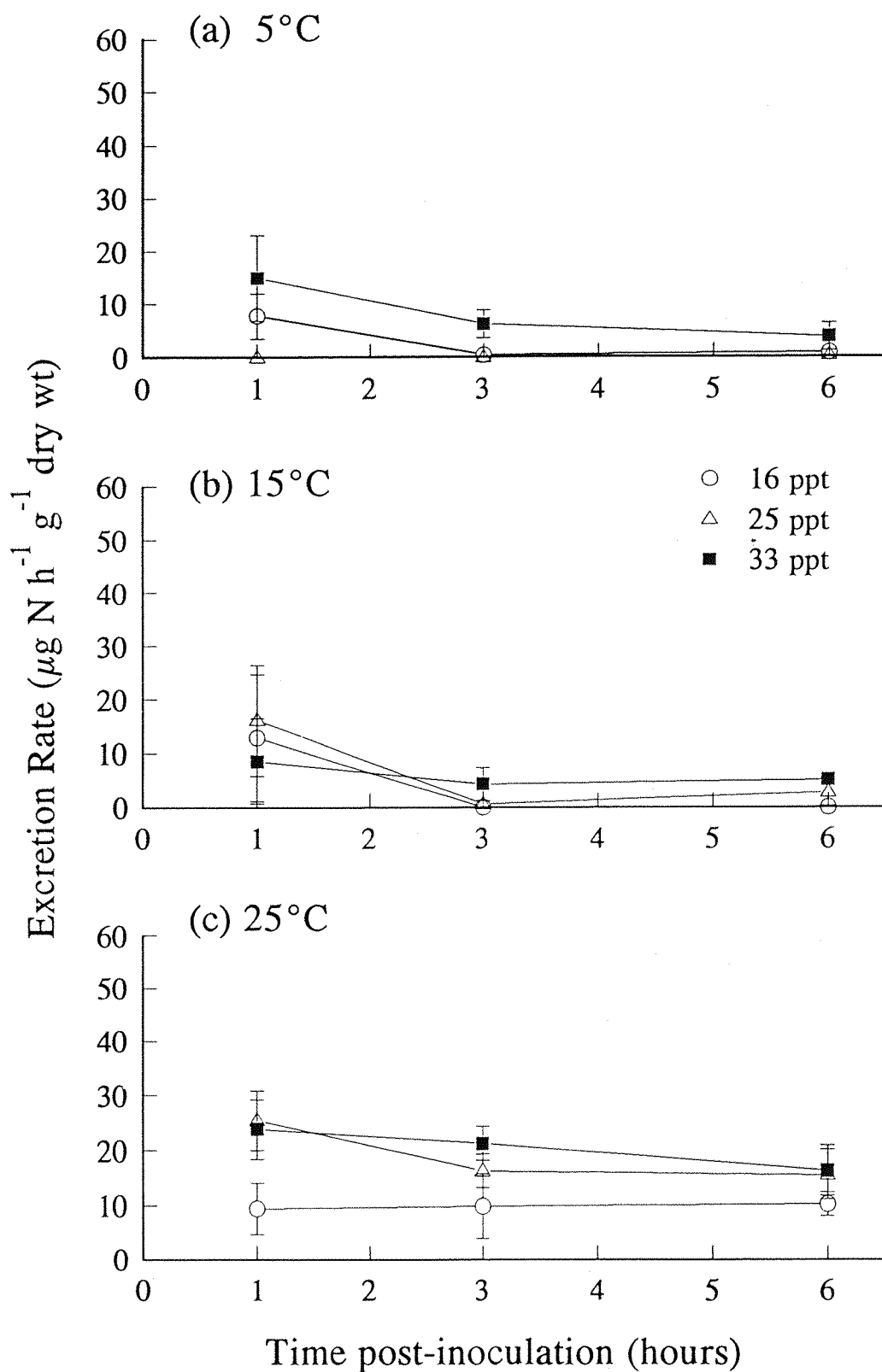


Figure 7.11 Control *Crassostrea gigas* (no bacteria). Effects of salinity on excretion rate ($\mu\text{g N h}^{-1} \text{g}^{-1} \text{dry weight}$) at three different temperatures. Mean \pm SE, n = 6 at each point.

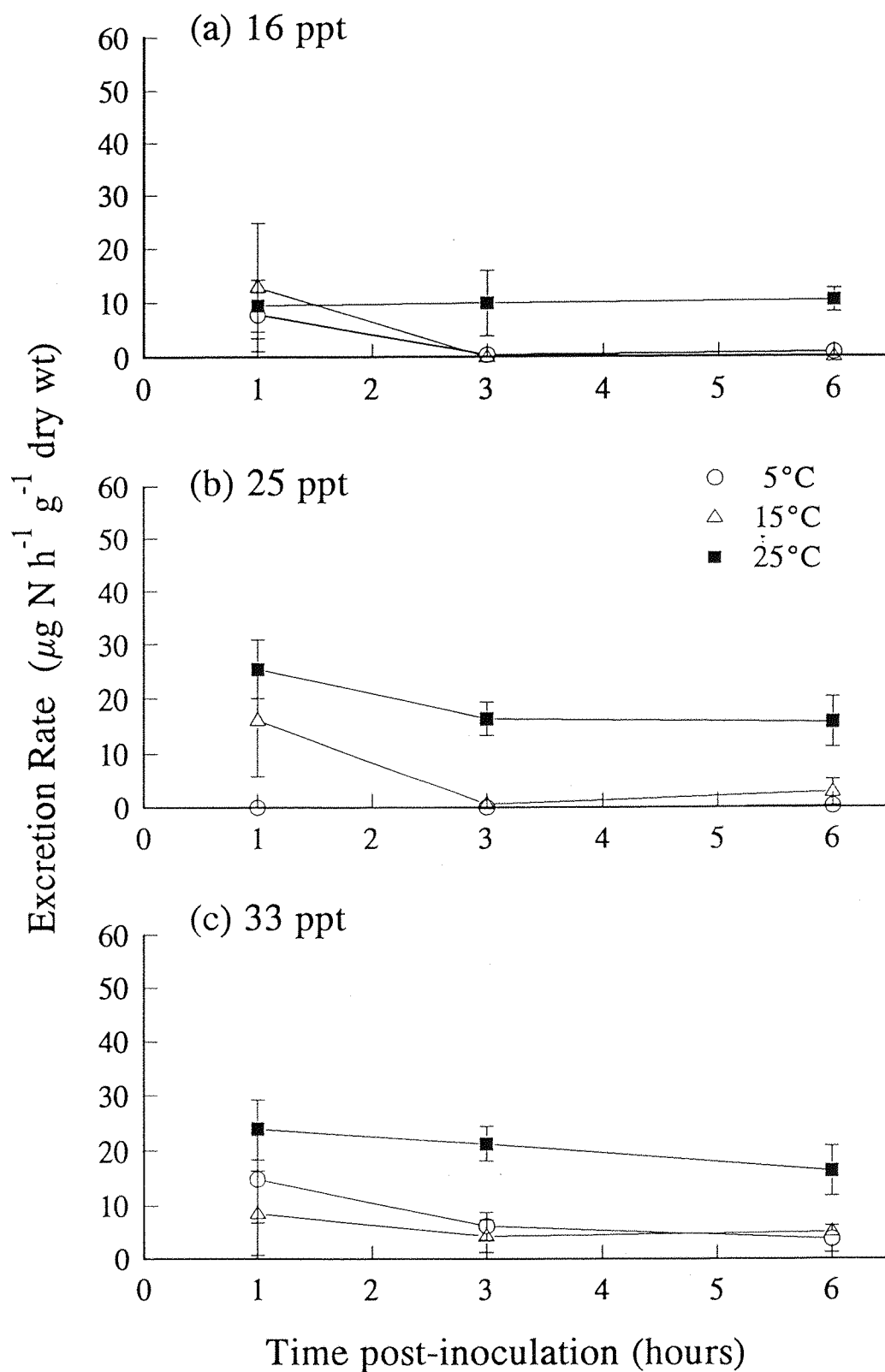


Figure 7.12 Control *Crassostrea gigas* (no bacteria). Effect of temperature on excretion rate ($\mu\text{g N h}^{-1} \text{g}^{-1} \text{ dry weight}$) at three different salinities. Mean \pm SE, n = 6 at each point.

Temp./Sal.	Time	Experimental	Control
5°C/16‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
5°C/25‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
5°C/33‰	1	n.s.	n.s.
	3	$P < 0.05$ (l)	n.s.
	6	n.s.	n.s.
15°C/16‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
15°C/25‰	1	n.s.	n.s.
	3	n.s.	$P < 0.05$ (l)
	6	$P < 0.05$ (l)	n.s.
15°C/33‰	1	n.s.	$P < 0.05$ (l)
	3	$P < 0.05$ (l)	$P < 0.05$ (l)
	6	n.s.	$P < 0.05$ (l)
25°C/16‰	1	$P < 0.05$ (l)	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
25°C/25‰	1	n.s.	n.s.
	3	n.s.	n.s.
	6	n.s.	n.s.
25°C/33‰	1	$P < 0.05$ (l)	n.s.
	3	$P < 0.05$ (l)	n.s.
	6	$P < 0.05$ (l)	n.s.

Table 7.04 Comparison of experimental and control results for excretion rate with baseline responses (Baseline data taken from chapter 2). Significant comparisons are shaded. n.s. = not significant ($P > 0.05$). (h) = higher than baseline, (l) = lower than baseline.

7.4 DISCUSSION

The temperature and salinity combinations chosen for these experiments were based on the upper and lower limits and the mid-points of the range used for determining baseline responses (chapter 2). This allowed direct comparisons to be made between the challenge and 'normal' figures recorded, as well as testing the effects of environmental extremes. The assays were begun before the monthly sampling data (chapter 6) was fully analysed, so when establishing these pathogen challenges it was unknown that there had been a lack of significant response until 48 hours after inoculation. Consequently, all the measurements were made over a six hour period following inoculation.

7.4.1 Hydrogen Peroxide Production

The data for hydrogen peroxide production in *Vibrio anguillarum* challenged *Crassostrea gigas* and in control animals are presented in figures 7.01-7.04. No obvious trends arose from the data, but it was found that at 25‰ and 33‰ the highest levels of peroxide production occurred at lower temperature. As the temperature increased, peroxide concentration decreased. This finding was similar to that in the monthly sampling of *Ostrea edulis*, presented in chapter 6 and also by Hawkins and Hutchinson (1990), when a decline in hydrogen peroxide production during the summer months was coincident with the main reproductive period.

Increases in bacterial diseases of oysters often occur during the summer months, as the temperature rises and animals undergo reproduction (Purdue, 1983; Beattie *et al.*, 1988), and a similar situation has been reported in *C. gigas* in Japan (Tamate *et al.*, 1965). Pacific oyster nocardosis is heavily influenced by environmental variables, although exactly how is unknown, but animals reared in cooler waters never show signs of the disease (Fisher *et al.*, 1992). The observed decrease, at high temperature, in hydrogen peroxide production in these *Vibrio* challenge experiments, supports these findings. Its unclear as to why this should be, but it may in part be a result of seasonal physiological changes. During the summer months, most energy has been shown to be directed towards

gametogenesis and reproduction, leaving little for the immunological processes. When the temperature does drop to winter conditions, energy is no longer put into reproductive mechanisms, but towards maintaining physiological (and immunological) competence. Salinity was shown to have less effect on physiology (chapter 2) although it probably has some effect on immunological processes (Fisher, 1988b; Chu and LaPeyre, 1989). For this study, the only significant salinity effects occurred at 15°C, when production of hydrogen peroxide at 25‰ was much lower than the other levels. This may well have been indicative of a changeover point in seasonal physiology, resulting in less energy available for immunological processes.

The control animals appeared to show less variation than animals challenged with *Vibrio anguillarum*, although there were no statistically significant differences. This suggests the possibility that the bacteria had some effect on hydrogen peroxide production, but not of sufficient magnitude to be accurately (or significantly) recorded. If the assay were extended to a 48 hour time period, as in the monthly challenge experiments of *Ostrea edulis*, then it is highly likely that significant changes would be observed, even allowing for a deterioration in water quality. The control animals showed significantly decreasing peroxide concentration with time at 5°C or 33°C. In both instances the conditions were not those described as optimum for *Crassostrea gigas* (chapter 2) so it is probable that the stress of maintaining physiological functions at these conditions means that less resources are available for immunology purposes, resulting in the decrease over time in hydrogen peroxide production.

When compared with baseline hydrogen peroxide production (Hawkins *et al.*, 1993b) the results for both control and bacterially challenged animals only varied significantly at 5°C, and only after one or three hours (table 7.01). In all cases these assays measured higher peroxide levels than were reported as 'normal'. That these maximum levels occurred during winter conditions under pathogenic challenge supports the hypothesis that animals at lower temperature are better able to cope with disease. The lack of any other significant differences suggests that the oysters were not unduly stressed during the first six hours of bacterial presence. By extending the time of the experiment, it could be determined whether certain

conditions are detrimental to the immunocompetence of the animal, or whether the physiological plasticity exhibited by *C. gigas* in chapter 2 means it is able to cope over a broad spectrum of variables.

7.4.2 Lysozyme Activity

The measurements of lysozyme activity in control and bacterially challenged *Crassostrea gigas* (figures 7.05-7.08) showed, as in chapter 6, a high degree of variability. This resulted in an absence of statistically significant differences within and between both sets of experimental animals. These assays were probably the most in need of a long incubation time, as demonstrated in the previous chapter, before any significant change could be detected, or it could be that these bacteria have no effect in raising lysozyme levels. Cheng *et al.* (1978) challenged the gastropod *Biomphalaria glabrata* with *Bacillus megaterium*, to investigate whether the introduction of bacteria into the mollusc induced hypersynthesis of lysosomal enzymes. The bacteria were injected directly into the snails, rather than incubating the host in an environment containing the pathogen. A significant increase in serum protein was observed in injected snails compared with controls, although this was not as a result of increased lysozyme or aminopeptidase, but was caused by undetermined serum protein fraction. Yoshino and Cheng (1976) reported that *Crassostrea virginica* haemolymph demonstrated significantly elevated aminopeptidase upon *in vitro* challenge with *B. megaterium*, thus showing that increased lytic enzyme synthesis under pathogen load is not necessarily represented by increased lysozyme production. It has also been reported that the presence of some bacteria can elicit a chemotactic response in molluscan haemocytes (Cheng and Guida, 1980; Cheng, 1988) but not in others (Cheng and Howland, 1979). These observations all show that enzyme measurement or haemolymph response to pathogenic stimulus can be very variable amongst bivalves, and is a possible explanation of the variability observed in these experiments. Besides extending the period of the experiment, it may also be desirable to measure other haemolymph protein activity, or to use different pathogens for challenge. These alternatives would help to provide more definite

reasons for the observed changes.

Some significant decrease in lysozyme activity was observed between 3 and 6 hours post-inoculation, at both 5°C and 33‰. This probably resulted from similar reasons as for hydrogen peroxide production, with energy required less for immunological processes and more for physiological maintenance. The low lysozyme activity at high salinity corresponds with the negative correlation proposed by Chu and LaPeyre (1993). Haemocyte activity and lysozyme concentration in *Crassostrea virginica* have been reported to change seasonally (Chu and LaPeyre, 1989; Fisher *et al.*, 1989), and to be affected by salinity (Fisher, 1988b; Chu and LaPeyre, 1989). Similar responses have been observed in a study of three geographic populations of *C. virginica*, under different salinity regimes, in response to *Perkinsus marinus* parasitism (Chu and LaPeyre, 1993). It was shown that animals from deeper water with low salinity had higher haemolymph cell numbers, a higher lysozyme concentration, and a better body condition index compared with animals from higher salinity regimes. Thus, these animals had a greater tolerance of infection as well as prolonged survival. Fisher *et al.* (1992) also showed that salinity, temperature and feeding all had important effects on *P. marinus* infection of *C. virginica*. Temperature was the dominant factor, with higher mortalities at elevated temperatures.

The control animals for the present study showed, as in the hydrogen peroxide assay, less variability (although not significantly so) than the experimentally challenged animals. This meant there were no statistically significant differences between any comparisons, but it may also suggest, again, that the bacterial challenge had some unidentified effect. However, when the control and challenge results were compared with baseline responses (Hawkins *et al.*, 1993b), the only significant differences found were at high temperature and salinity after 3 hours (table 7.02). Some of these animals gave no recordable lysozyme activity, resulting in much lower levels than the baseline responses. That these occurred at high temperature is indicative of the summer physiology providing less energetic resource for immunological purposes. There is also probably a high degree of inherent variability in measurements of individual animals.

7.4.3 Excretion Rates

The results for ammonia excretion in bacterially challenged and control groups are presented in figures 7.09-7.12. This physiological index was used to measure whether there was any effect of maintaining the animals in an enclosed environment for six hours, and also to indicate whether the challenge compromised both oyster physiological and immunological processes.

Excretion in challenged oysters at 5°C/16‰, after 1 hour post-inoculation, was the highest rate recorded, but was not significantly different from the rates recorded for these conditions at 3 and 6 hours post-inoculation. This again reflected inherent variability between individual animals. The significant difference between control and challenge animals at 5°C/25‰ was a result of the lack of excretion detected in the control animals. At several combinations of temperature and salinity measured, no excretion was recorded, suggesting the animals remained shut, possibly owing to disturbance or to the presence of bacteria in the environment. This may also explain the lack of enzyme activity detected in some animals.

Changing environmental conditions have been shown to alter excretion rate in *Ostrea edulis* (Hutchinson and Hawkins, 1992), with large variations observed. For the flat oyster, as with *Crassostrea gigas* (chapter 2) temperature was shown to be the major influencing factor, although salinity may also influence the turnover of nitrogenous compounds related to osmoregulation (Prosser, 1973; Hutchinson and Hawkins, 1992). It was also indicated for *C. gigas* that excretion rate frequently reflected the health of the animal, as animals with a reduced body condition index also had a higher nitrogenous output (Hawkins *et al.*, 1993b). Parasitism of oysters has also been shown to result in reduced physiological processes. Wilson *et al.* (1988) found that *Boonea impressa* infection in *Crassostrea virginica* caused a significant decline in growth rate, reproductive development, and net productivity, although the oysters retained a net positive energy balance. Unfortunately, the authors did not ascertain whether these results arose because of a reduced filtration rate, resulting in less energy being assimilated, or by the direct removal of assimilated energy by the parasite.

Comparison of measurements made at 1 hour post-inoculation showed that

excretion rates measured at 5°C/16‰ and 15°C/33‰ were significantly higher than all the others. It is unclear why this should be, but it could be a reflection of seasonal physiological differences. Under these temperature and salinity conditions, the animals are able to maintain their normal metabolic and physiological functioning, at least for the first hour after inoculation. As time progressed to 3 and 6 hours after inoculation, the excretion rate fell to similar levels as measured for the other conditions. The variability observed was again probably a reflection of the inherent differences in each individual animal. The significant differences recorded by two-way ANOVA were also probably a result of the lack of excretion rate measured in certain animals, which may well be the result of animals remaining closed. Those differences which did occur, as in chapter 2, were mainly a result of temperature changes, with salinity having little effect on the physiology.

The results for control animals were again less variable than those for the challenged animals, although not statistically significantly so. These excretion rates, apart from being less variable, were generally lower than for the other experimental animals, which suggests, as for the hydrogen peroxide and lysozyme assays, that the bacterial challenge had some effect on *C. gigas*. Unfortunately, it is still difficult to ascertain what that effect was. The only significant effect which did occur was at 5°C, where there was no recorded excretion for animals at 25‰. This is also very difficult to explain, especially in the control animals, but it may be a result of minimal winter physiological activity. This lack of excretion measured in some animals meant that, when these results were compared with the normal, baseline responses of chapter 2, many of these values were lower, perhaps indicating the oysters were remaining closed in response to pathogen presence. In general, the excretion rate being a physiological process, appeared to be influenced more by temperature than salinity, and tended to exhibit a decreased rate with time.

7.4.4 Summary

Overall, there were no obvious trends for any of the indices measured. If

the experimental period had been extended to 48 hours then this may have changed, and a more definite response to pathogenic challenge observed (see chapter 6). For each of these three indices the control animals, incubated with broth but no bacteria, appeared to be less variable than the bacterially challenged oysters, although no set of comparisons was statistically significant.

Hydrogen peroxide production showed the highest levels occurring at low temperature (5°C), supporting observations made in chapter 6. As the temperature increased, peroxide activity decreased, probably as a result of associated increase in reproductive and gametic maturation, and their use of energetic resources. Control animals were less variable, with decreasing peroxide levels over time at 5°C and at 33‰. This probably resulted from physiological stress at non-optimum conditions. Comparisons with baseline responses showed significant differences only occurred at 5°C, probably as a result of the energetic resources at this temperature being available for immunological purposes.

Lysozyme measurements were highly variable resulting in no significant variation over the conditions measured. This index, more than the others, probably required measuring over 48 hours to enable a proper response to occur and be recorded. At 5°C and at 33‰ there was a significant decrease in enzyme activity from 3 to 6 hours post-inoculation, probably as a result of energy partitioning as for hydrogen peroxide. Control animals were less variable but with no significant differences. The results for lysozyme activity after bacterial challenge were lower than the baseline levels as a result of inherent differences in individuals, with some oysters having no detectable activity.

Excretion rates were mainly influenced by temperature, with rates tending to decrease after 3 to 6 hours post-inoculation. This was probably a result of the animals remaining shut. Control animals had lower, less variable rates of excretion, but these differences were non-significant. The only significant differences between control results occurred at 5°C, probably as a result of winter physiology leading to minimum physiological activity. When compared with baseline responses, some of these challenge results were significantly lower, reflecting the valves remaining shut in response to the pathogen, and thus having little or no detectable excretion.

CHAPTER EIGHT

GENERAL CONCLUSIONS AND FUTURE PROPOSALS

CHAPTER 8

GENERAL CONCLUSIONS AND FUTURE PROPOSALS

The original remit of this project was to examine the extent to which stress, when imposed on an organism, caused a change in the health of that organism, and its resistance or susceptibility to possible disease. When studying disease in animals, there is an underlying need to know what the natural response of the host is to a given set of conditions, before any changes in this response can be regarded as abnormal. This knowledge is required at several different levels of the animals' organisation, such as its physiological, immunological and metabolic processes, as well as other areas of its life cycle, such as its feeding and reproductive strategies. Although there are a multiplicity of factors which can elicit stress responses, the intrinsic elements of temperature and salinity were the major study variables used. The study organisms of this project were largely represented by two species, the Pacific oyster *Crassostrea gigas* and the European flat oyster *Ostrea edulis*, but additional information was obtained by utilising the Manila clam *Tapes philippinarum*. The major aim of this study was initially to provide baseline measurements of physiology and immunology, and to a lesser extent metabolic processes, across a matrix of environmental conditions, thus providing a synopsis of the 'natural' behaviour of these animals. The study then proceeded to examine how these responses changed when the animal was under pathogenically induced stress. In addition, the use of enzyme electrophoresis allowed some quantification of genetic differences between three populations of *Ostrea edulis*.

The quantification of physiological responses to environmental change was made by an index of scope for growth (SFG). This measured filtration, respiration and excretion rates, and the digestive absorption efficiency of the animals. In all the SFG indices quantified for each of the three species, the overwhelmingly dominant factor for each proved to be the filtration rate. It is filtration of food

from the water column which determines the potential amount of energy available to the animal for growth and reproduction, so it is hardly surprising that this was the case. In *Crassostrea gigas* the optimal rates for this were at high temperature (20-25°C) and mid-salinity (19-25‰), indicating this to be derived from a warm water, estuarine stock. Similar conditions in *Ostrea edulis* proved to be highly detrimental, with the optima for this species occurring at more marine salinities. Application of this knowledge to the commercial culture of oysters would perhaps allow successful production of both species in an estuarine site, with *C. gigas* grown higher up the estuary than *O. edulis*, but with both species having a rapid growth rate and high meat yield.

These measurements allow species to be compared directly, because acclimation procedures and food quality were the same for both. It was also interesting to see how the stress of temperature or salinity shock affected these animals, especially their susceptibility to disease. Filtration in bivalves is known to be highly sensitive to other environmental parameters such as water flow rates, suspended particulate concentrations, and the constituent parts of this suspended matter (whether organic or inorganic).

The effect of tidal exposure was only examined briefly in *O. edulis*, and showed that the accumulation of lactate can be particularly harmful to haemocytic defence mechanisms. Further investigation into how lactic acid actually inhibits haemocyte movement is required; it would also be useful to investigate how lactate affects associated immunological processes, such as hydrogen peroxide production, and also the effects of exposure at different temperatures, and the recovery period required to regain 'normal' immunological efficiency.

Measurements of respiration rates in both *C. gigas* and *O. edulis* provided the clearest indication of apparent separate winter and summer states. This change occurred at 10-12°C in *O. edulis* and at 15°C in *C. gigas*, indicating the seasonal range normally inhabited by these animals. With a higher oxygen demand at higher temperature, it would be expected that hypoxia induced by tidal exposure would be more detrimental at higher temperatures than lower ones. The switch in seasonal physiology which occurs at a higher temperature in *C. gigas* compared with *O. edulis*, suggests it would be interesting to determine how each species

cope with anaerobiosis and lactic acid accumulation at a range of temperatures.

When the scope for growth indices of the two oysters were compared, it was found that *Crassostrea gigas* functioned much better under the experimental conditions used than *Ostrea edulis*. Under some circumstances, *O. edulis* demonstrated negative scope for growth, where it was using up stored reserves, and even suffered some mortality. *C. gigas* did not present either of these problems suggesting the Pacific oyster is a more than adequate commercial replacement.

The use of a body condition index (BCI) showed an inverse relationship with SFG. This was probably a reflection of the reproductive cycle, where spawning resulted in a dramatic reduction in body tissue, thus reducing the BCI. Since SFG did not distinguish between reproductive and somatic growth, the use of a gonadal index would have been useful. It is therefore clear that the age, size and stage of maturity of oysters are all important factors in determining the SFG and body condition indices of the animals, and consequently their energetics and ability to resist disease.

Examination of the way in which *C. gigas* partitioned its energetic resources demonstrated that this species was well able to adapt to prevailing conditions. At low temperatures, the basic metabolic processes were maintained with minimal energy uptake and usage. As the temperature increased, firstly the energy uptake increased and later the use of that energy increased, indicating that it is environmental factors, and particularly temperature, which affect the amount of energy assimilated by the oyster, and the way this is ultimately used.

The information provided by *Tapes philippinarum* was useful because it demonstrated that, although under the same regime as *O. edulis* and *C. gigas*, this bivalve exhibited subtly different responses. As has been mentioned, filtration rate was considered the predominant influencing factor, but for this, and the other rates measured there was no linear relationship with temperature in *T. philippinarum*. Unfortunately, there was not as much data for this species as for the Pacific oyster, so it is difficult to determine whether any seasonal physiologies exist. There is a large change in filtration, and respiration and excretion rates, between 10°C and 15°C, which suggests that any switch from winter to summer

physiology could occur at this point. However, *Tapes philippinarum* is derived from a warm water stock, as is *C. gigas*, so it would be expected that any seasonal physiology change would occur at a temperature more similar to that of *C. gigas* than *O. edulis*. *T. philippinarum* may have become rapidly adapted to more temperate waters, an adaptability and 'hardiness' which could be very valuable if exploited by the commercial market. Further investigations, for example with enzyme electrophoresis, are required to determine whether the physiological and genetic make-up of this species can alter in relatively few generations.

The partitioning of energy, and the uptake and usage of that energy, demonstrated that *T. philippinarum* adapted and acclimated readily to the conditions imposed on it, at least at high salinity. Further investigations covering the additional salinity range used for the oyster investigations would provide more detailed information as to how this species could cope with the conditions encountered in coastal and estuarine bivalve culture. The effects of salinity would be particularly interesting as it is this environmental parameter which is likely to have the most significant influence on the haemocytic indices quantified for *T. philippinarum*. Temperature appeared to have little effect on these indices, although there was a significant increase in haemocyte numbers from 5°C to 10/15°C, possibly as a result of seasonal physiologies.

The results obtained for these bivalve species indicate that high temperatures result in gametogenesis, thereby reducing BCI and haemocyte numbers, and causing a drain on resources through spawning. This would also make the animals more susceptible to disease. For the oysters, these factors must be balanced against the increased levels of filtration, and therefore SFG, which occur at high temperatures, and might provide some energy for disease resistance. At low temperatures, animals tend to maintain their basal metabolism and haemocyte functions at a minimum, although for *O. edulis* the filtration rate may be reduced to the extent it results in negative SFG and the use of storage products.

Following the establishment of normal baseline responses to environmental conditions, it was possible to determine how they were affected by the presence of pathogenic organisms. The use of a site known to have been infected by the

protozoan parasite *Bonamia ostreae*, an organism which devastated much of the European *O. edulis* fishing industry during the last twenty years, provided ideal conditions for the study. The use of three geographically isolated populations of *Ostrea edulis* enabled a comparative study of physiological plasticity to be established. As has already been stated, filtration rate is accepted to most influence the SFG index. However, there were a number of apparently anomalous observations made (Hawkins *et al.*, 1993c). It has previously been recorded that filtration rate increases with size (Winter *et al.*, 1984) whereas the present study recorded an inverse size-filtration rate relationship. Some of the differences in filtration rates measured were not significant, which may have been a result of a variety of reasons, acting independently or synergistically. The most obvious problem with the experimental design was the difference in size, and consequently age and sexual maturity, of the populations. The results for *Crassostrea gigas* and *Tapes philippinarum* have already suggested that the reproductive cycle is highly influential to the BCI and SFG, depending on when measurements are made. Another problem was caused by the slightly different sampling times of the oysters, with a fourteen month gap between each field trip resulting in slightly different seasonal effects. Ideally the study should have been performed (or repeated) with animals of the same size and age, and with sampling occurring in both winter and summer conditions, over a two year cycle.

The extent to which individual animals were parasitised was also difficult to ascertain. For some of the changes in physiological rates noted, it was not clear whether the degree of parasitism recorded for a population occurred as a result of environmental influence causing a change in physiology, or whether the degree of parasitism actually caused the changes observed. Field studies on their own are insufficient to establish the extent of each possible cause of change, so careful laboratory studies are needed. These would be able to control the numbers of parasites the animals are exposed to, and the length of time each oyster is exposed for, and under what environmental conditions. The resulting SFG measurements in the present field trial showed that the Scottish oysters had increased their energetic resources, thereby increasing their potential for fighting disease, which ultimately lead to their decreased levels of parasitism recorded. In contrast, the Welsh

oysters had a decreased potential for pathogenic resistance. Comparison of the energy partitioning of each population showed that the smallest animals had the highest amounts of energy partitioned into SFG. The larger animals had less of their assimilated energy for somatic or gametogenic growth, but still maintained a degree of resistance. Thus, it is not the amount of energy which determines susceptibility or resistance, but the actual fate of that energy. This requires the measurement of other processes (included in Hawkins *et al.*, 1993b; 1993c), especially haemocytic and immunological indices, in order to determine which of these processes is benefitting most from assimilated energy.

In an effort to minimise the seasonal effects of sampling time, the experiments conducted in the laboratory were maintained under the same conditions for both years. In this case it may therefore be assumed that the variability measured was as a result of inherent differences between the populations. This could have been caused by phenotypic plasticity demonstrated by different populations, but may also have been caused by the differences in size, age and sexual maturity (*vide* van Banning, 1991).

An investigation into the enzymic variation between the populations was carried out to determine if there was any genetic basis for differences or similarities encountered. *O. edulis* has previously been described as having low genetic variability (Saavedra *et al.*, 1993). The lack of significant variation in indices such as the number of alleles per locus suggested that a similar situation was present in these populations. The amount of polymorphism within the three groups was found to be similar to a previous study of non-sibling oysters (Buroker, 1982), and when compared with other molluscan species, *O. edulis* was found to have very low genetic variation. This suggests that any differences in disease resistance between the populations occurred as a result of different combinations of physiological, immunological, or metabolic acclimation to the conditions, and was not genetic in nature.

Further examination showed the three populations to be 90-100% identical at more than three-quarters of their loci, and that it was only at two loci where major variation was found. Additional, more extensive studies are required, using a greater number of animals, from more sites, and with a larger number of loci

observed. Loci involved with specific parts of the metabolic pathway could be used in an effort to determine where any selective pressure may be taking place. At present though, this study has to conclude that there is little or no genetic basis for observed physiological differences in the three British populations.

The field investigations of *O. edulis* were extended further by examining seasonal changes in immunocompetence. Pathogenic challenge was introduced, to determine whether animals were more susceptible to infection at certain times. Experiments investigating phagocytosis found there was no significant correlation with any of the environmental parameters measured, although increased levels did occur when particulate matter increased. This was most probably a result of bacteria associated with this material, either flocculated within the suspended particulates or adhered to their surface. This would suggest that temperature has less effect than turbulence and re-suspension, in promoting bacterial disease. This would be further compounded by the occurrence of phytoplankton blooms which can lead to associated bacterial blooms. Additional evidence to support this theory would be useful, and could be obtained by extending the length of the study, and making phytoplankton and bacterial counts. Laboratory based experiments measuring suspended particulates, including organic and inorganic matter, and their effects on immunological and physiological parameters, would also be useful. This would require slight modification of the experimental protocol, so that phagocytic and immunological measurements are both made *in vitro*.

Hydrogen peroxide and lysozyme production did not correlate significantly with environmental variables, although both showed minimal activity during the summer. This coincided with the brooding period, where resource partitioning had probably redirected energy from immunological processes to reproductive ones. It is possible that this is linked with seasonal physiology, as maximum production levels occurred in winter, and indicates that temperature has an indirect effect on oyster immunology by promoting gametogenesis.

The use of *Vibrio anguillarum* challenge showed that hydrogen peroxide is the primary immunological defence mechanism in *O. edulis*. The length of time required for an increase to be detected was probably a result of the methodology used. Although *Vibrio anguillarum* is a fast growing bacterium, it is unlikely that

numbers flourished excessively owing to the lack of additional nutrients. As a result the delay probably occurred because of the length of time take to infiltrate the oyster tissues.

Pathogenic challenge of *Crassostrea gigas* was undertaken under controlled laboratory conditions using different temperature and salinity combinations. This study found similar results to the challenge experiments with *Ostrea edulis* from the Solent, whereby hydrogen peroxide appeared to be the primary defence mechanism. The lysozyme results proved to be very variable. Unfortunately, these assays were only performed over a 6 hour period and not over 48 hours as for *O. edulis*. If the experimental time was extended then it now appears likely that significant trends would arise as the bacteria have time to infiltrate the tissues properly.

Hydrogen peroxide levels in *C. gigas* were again highest at low temperatures, lending further supportive evidence that reproduction at high temperatures results in less energy being available for immunological processes. At lower, winter temperatures this is reversed. The controls for these experiments were more thorough than for the *O. edulis* challenges and suggested that, although no significant differences were observed, the bacteria probably did influence hydrogen peroxide production. This also appeared to be the case in the lysozyme activity and excretion rate experiments. The variability in lysozyme results meant no significant differences were found; however, it was discovered that not all enzyme production in response to pathogenic challenge is in the form of lysozyme activity. Modifications to the experimental protocol could include the measurement of different haemolymph proteins, as well as the use of alternative haemocytic indices such as cell numbers, rate of locomotion and cell size change. It may also be useful to utilise different pathogens to challenge the bivalves, to use different concentrations of bacteria, or to change the method of challenge, e.g. by injecting the bacteria directly into the hosts. While this procedure would alleviate the problem of the time taken for bacteria to infiltrate the tissues, it is not representative of natural uptake of pathogens.

Excretion rate measurements in *C. gigas* showed similar responses to temperature and salinity influences as in the baseline measurements, with

temperature having the most significant effect. The quantification of other physiological processes would also be useful to determine what effect pathogenic challenge has on scope for growth and energy partitioning. This would also allow comparisons with the field trials of the three *Ostrea edulis* populations, possibly providing better explanations of what is occurring, as well as contributing quantitative data under different environmental conditions. The baseline data already established provided worthwhile comparisons with the bacterial challenge measurements, although they did not demonstrate vast differences.

This study has provided valuable data on the natural physiological behaviour of three bivalve species, both under variable environmental conditions and with the burden of pathogenic load. Field samples have demonstrated the inherent variability of native oysters under normal seasonal conditions, and have allowed comparison with studies of this species, as well as the Pacific oyster and the Manila clam, in the controlled environment of the laboratory. The nature of these stressors, and the way in which they effect the susceptibility and resistance to disease of molluscs has been determined, whilst some study has been made of underlying genetic processes. These results may be used as a means of predicting the suitability of new sites or species for cultivation, or to determine the effects of environmental change in existing habitats. The assays have also led to suggestions of future avenues of research. Altogether, the information presented in this study provides a comprehensive and valuable insight into molluscan responses to natural environmental variability, and their effects on disease processes.

LITERATURE CITED

LITERATURE CITED

- ADAMKEWICZ, L., TAUB, S.R. and WALL, J.R. (1984). Genetics of the clam *Mercenaria mercenaria*. 2. Size and genotype. *Malacologia*, **25**; pp. 525-533.
- AHMED, M. (1975). Speciation in living oysters. *Adv. Mar. Biol.*, **13**; p. 357.
- ALLEN Jnr, S.K., DOWNING, S.L. and CHEW, K.K. (1989). *Hatchery Manual for Producing Triploid Oysters*. University of Washington Press, Seattle, Washington. 27 pp.
- ALVAREZ, M.R., FRIEDL, F.E., HUDSON, C.M. and O'NEIL, R.L. (1992). Effects of hypoxic and hyperoxic conditions on hemocyte activity and abiotic particle retention by the eastern oyster, *Crassostrea virginica* (Gmelin, 1791). *J. Shell. Res.*, **11**; pp. 383-386.
- ANDREWS, J.D. and RAY, S.M. (1988). Management strategies to control the disease caused by *Perkinsus marinus*. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 257-264.
- AUFFRET, M. (1988). Bivalve haemocyte morphology. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 169-177.
- AYALA, F.J., TRACEY, M.L., HEDGECOCK, D. and RICHMOND, R.C. (1975). Genetic differentiation during the speciation process in *Drosophila*. *Evolution*, **28**; pp. 576-592.
- BACHERE, E., HERVIO, D. and MIALHE, E. (1991). Luminol-dependent chemiluminescence by hemocytes of two marine bivalves, *Ostrea edulis* and *Crassostrea gigas*. *Dis. Aquat. Org.*, **11**; pp. 173-180.
- BALLANTYNE, J.S. and BERGES, J.A. (1988). The effects of salinity acclimation on the metabolism of the oyster *Crassostrea virginica*. *Amer. Zool.*, **28**; abstract.
- BALLANTYNE, J.S. and BERGES, J.A. (1991). Enzyme activities of gill, hepatopancreas, mantle, and adductor muscle of the oyster (*Crassostrea virginica*) after changes in diet and salinity. *Canad. J. Fish. Aquat. Sci.*, **48**; pp. 1117-1123.

- BALLANTYNE, J.S. and MOYES, C.D. (1987). The effects of salinity acclimation on the osmotic properties of mitochondria from the gill of *Crassostrea virginica*. *J. Exp. Biol.*, **133**; pp. 449-456.
- BALOUET, G., PODER, M. and CAHOUR, A. (1983). Haemocytic parasitosis : morphology and pathology of lesions in the French flat oyster, *Ostrea edulis* L. *Aquaculture*, **34**; pp. 1-14.
- BANNISTER, R.C.A. and KEY, D. (1982). *Bonamia*, a new threat to the native oyster fishery. *Fish. Not.*, *MAFF Direct. Fish. Res.*, *Lowestoft*, **71**; 9 pp.
- BARBER, B.J. and BLAKE, N.J. (1981). Energy storage and utilization in relation to gametogenesis in *Argopecten irradians concentricus* (Say). *J. Exp. Mar. Biol. Ecol.*, **52**; pp. 121-134.
- BARBER, B.J., FORD, S.E. and HASKIN, H.H. (1988a). Effects of the parasite MSX (*Haplosporidium nelsoni*) on oyster (*Crassostrea virginica*) energy metabolism. I. Condition index and relative fecundity. *J. Shell. Res.*, **7**; pp. 25-35.
- BARBER, B.J., FORD, S.E. and HASKIN, H.H. (1988b). Effects of the parasite MSX (*Haplosporidium nelsoni*) on oyster (*Crassostrea virginica*) energy metabolism. II. Tissue biochemical composition. *Comp. Biochem. Physiol.*, **91**; pp. 603-608.
- BARBER, B.J., FORD, S.E. and LITTLEWOOD, D.T.J. (1991). A physiological comparison of resistant and susceptible oysters *Crassostrea virginica* (Gmelin) exposed to the endoparasite *Haplosporidium nelsoni* (Haskin, Stauber & Mackin). *J. Exp. Mar. Biol. Ecol.*, **146**; pp. 101-112.
- BARILLÉ, L., PROU, J., HÉRAL, M. and BOURGRIER, S. (1993). No influence of food quality, but ration-dependent retention efficiencies in the Japanese oyster *Crassostrea gigas*. *J. Exp. Mar. Biol. Ecol.*, **171**; pp. 91-106
- BAROSS, J. and LISTON, J. (1968). Isolation of *Vibrio parahaemolyticus* from the northwest Pacific. *Nature*, **217**; pp. 1263-1264.
- BAROSS, J. and LISTON, J. (1970). Occurrence of *Vibrio parahaemolyticus* and related hemolytic vibrios in marine environments of Washington State. *Appl. Micro.*, **20**; pp. 179-186.

- BAYNE, B.L. (ed.) (1976). *Marine Mussels: Their Ecology and Physiology*. Cambridge University Press, Cambridge, U.K.
- BAYNE, B.L. (1984). *Response to Environmental Stress*, in Proceedings 18th European Marine Biology Symposium, Oslo. (Eds.) Gray, J. and Christenson, M. Wiley, New York.
- BAYNE, B.L. (1993). *Feeding Physiology of Bivalves : Time-Dependence and Compensation for Changes in Food Availability*, in Bivalve Filter Feeders in Estuarine and Coastal Ecosystem Processes. (Ed.) Dame, R.F. Springer-Verlag, Berlin. pp. 1-24.
- BAYNE, B.L., BROWN, D.A., BURNS, K., DIXON, D.R., IVANOVICI, A., LIVINGSTONE, D.R., LOWE, D.M., MOORE, M.N., STEBBING, A.R.D. and WIDDOWS, J. (1985). *The Effects of Stress and Pollution on Marine Animals*. Praeger, London. 384 pp.
- BAYNE, B.L., BUBEL, A., GABBOTT, P.A., LIVINGSTONE, D.R., LOWE, D.M. and MOORE, M.N. (1982). Glycogen utilization and gametogenesis in *Mytilus edulis* L. *Mar. Biol. Lett.*, 3; pp. 89-105.
- BAYNE, B.L., GABBOTT, P. and WIDDOWS, J. (1975). Some effects of stress in the adult on the eggs and larvae of *Mytilus edulis* L. *J. Mar. Biol. Assoc. U.K.*, 55; pp. 675-689.
- BAYNE, B.L., GEE, J.M., DAVEY, J.T. and SCULLARD, C.J. (1979). Physiological responses of *Mytilus edulis* L. to parasitic infestation by *Myticola intestinalis*. *J. Cons. Int. Explor. Mer.*, 38; pp. 12-17.
- BAYNE, B.L., HAWKINS, A.J.S. and NAVARRO, E. (1987). Feeding and digestion by the mussel *Mytilus edulis* L. (Bivalvia:Mollusca) in mixtures of silt and algal cells at low concentration. *J. Exp. Mar. Biol. Ecol.*, 111; pp. 1-22.
- BAYNE, B.L., HOLLAND, D.L., MOORE, M.N., LOWE, D.M. and WIDDOWS, J. (1978). Further studies on the effects of stress in the adult on the eggs of *Mytilus edulis*. *J. Mar Biol. Assoc. U.K.*, 58; pp. 825-841.
- BAYNE, B.L. and NEWELL, R.C. (1983). *Physiological Energetics of Marine Molluscs*, In : Saleuddin, A.S.M. and Wilbur, K.M. (Eds.) *The Mollusca : Physiology*, Vol 4 (1). Academic Press, New York. pp. 407-415.

- BAYNE, B.L., THOMPSON, R.J. and WIDDOWS, J. (1976). *Physiology : I*, in *Marine Mussels : Their Ecology and Physiology*. (Ed.) Bayne, B.L. Cambridge University Press, Cambridge, England. pp. 121-206.
- BAYNES, S.M., EMERSON, L. and SCOTT, A.P. (1979). *Production of Algae for Use in the Rearing of Larval Fish*. Fisheries Research Technical Report No. 53, Ministry of Agriculture, Fisheries and Food, Directorate of Fisheries Research.
- BEATTIE, J.H., DAVIS, J.P., DOWNING, S.L. and CHEW, K.K. (1988). Summer mortality of Pacific oysters. *Amer. Fish. Soc. Spec. Publ.*, 18; pp. 265-268.
- BEAUMONT, A.R. (1986). Genetic aspects of hatchery rearing of the scallop *Pecten maximus* (L). *Aquaculture*, 57; pp. 99-110.
- BEAUMONT, A.R., BEVERIDGE, C.M. and BUDD, M.D. (1983). Selection and heterozygosity within single families of the mussel *Mytilus edulis* (L). *Mar. Biol. Lett.*, 4; pp. 151-161.
- BEAUMONT, A.R. and KHAMDAN, S.A.A. (1991). Electrophoretic and morphometric characters in population differentiation of the pearl oyster, *Pinctada radiata* (Leach) from around Bahrain. *J. Moll. Stud.*, 57; pp. 433-441.
- BENINGER, P.G. and LUCAS, A. (1984). Seasonal variations in condition, reproductive activity, and gross biochemical composition of two species of adult clam reared in a common habitat, *Tapes decussatus* and *Tapes philippinarum*. *J. Exp. Mar. Biol. Ecol.*, 79; pp. 19-37.
- BERG, J. and NEWELL, R.I.E. (1986). Temporal and spatial variations in the composition of seston available to the suspension feeder *Crassostrea virginica*. *Est. Coast. Shelf Sci.*, 23; pp. 375-386.
- BERNARD, F.R. (1974). Particle sorting and labial palp function in the Pacific oyster *Crassostrea gigas* (Thunberg, 1795). *Biol. Bull.*, 146; pp. 1-10.
- BERNARD, R.F. (1983). Physiology and the mariculture of some Northeastern Pacific bivalve molluscs. *Can. Spec. Publ. Fish. Aquat. Sci.*, 63.
- BERRIGAN, M.E. (1990). Biological and economical assessment of an oyster resource development project in Apalachicola Bay, Florida. *J. Shell. Res.*, 9; pp. 149-158.

- BODOY, A., RIVA, A. and MAITRE-ALLAIN, T. (1986). Comparaison de la respiration chez *Ruditapes decussatus* (L.) et *R. philippinarum* (Adams & Reeve) en fonction de la température. *Vie Milieu*, **36**; pp. 83-89.
- BOURGRIER, S., GAGUENES, G., BACHERE, E., TIGE, G. and GRIZEL, H. (1986). Essai de réimplantation de *Crassostrea angulata* en France : résistance au chambrage et comportement des hybrides *C. angulata* - *C. gigas*. *ICES Shellfish Committee, K*, **36**; p. 14.
- BOWER, S.M. (1988). Circumvention of mortalities caused by Denman Island oyster disease during mariculture of Pacific oysters. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 246-248.
- BOWER, S.M. (1992). Winter mortalities and histopathology in Japanese Littlenecks [*Tapes philippinarum* (A. Adams and Reeve, 1850)] in British Columbia due to freezing temperatures. *J. Shell. Res.*, **11**; pp. 255-263.
- BRETT, J.R. (1958). *Implications and Assessments of Environmental Stress*, in *Investigations of Fish-Power Problems*. (Ed.) Larkin, P.A. University of British Columbia Press, Vancouver. pp. 69-83.
- BRICELJ, V.M., FORD, S.E., BORRERO, F.J. PERKINS, F.O., RIVARA, G., HILLMAN, R.E., ELSTON, R.A. and CHANG, J. (1992). Unexplained mortalities of hatchery reared, juvenile oysters, *Crassostrea virginica* (Gmelin). *J. Shell. Res.*, **11**; pp. 331-347.
- BROOKS, S. (1989). *Tidal Stress Effects on Defence Mechanisms of Mercenaria mercenaria*. BSc. Undergraduate Project, University of Southampton.
- BROWN, J.R. (1988). Multivariate analyses of the role of environmental factors in seasonal and site-related growth variation in the Pacific oyster *Crassostrea gigas*. *Mar. Ecol. Prog. Ser.*, **45**; pp. 225-236.
- BROWN, J.R. and HARTWICK, E.B. (1988a). Influences of temperature, salinity and available food upon suspended culture of the Pacific oyster, *Crassostrea gigas* .1. Absolute and allometric growth. *Aquaculture*, **70**; pp. 231-251.
- BROWN, J.R. and HARTWICK, E.B. (1988b). Influences of temperature, salinity and available food upon suspended culture of the Pacific oyster, *Crassostrea gigas* .2. Condition index and survival. *Aquaculture*, **70**; pp. 253-267.

- BUCKE, D. (1988). Pathology of bonamiasis. *Parasitology Today*, 4; pp. 174-176.
- BUCKE, D. and FEIST, S. (1985). *Bonamiasis in the flat oyster, Ostrea edulis, with comments on histological techniques*, in Fish and Shellfish Pathology. (Ed.) Ellis, A.E. Academic Press, London. pp. 387-392.
- BURKLEW, M.A. (1971). *Effects of Elevated Temperatures on Glycogen and Protein Fluctuations in Oysters*. In : A Preliminary Investigation : The Effect of Elevated Temperature on the American Oyster *Crassostrea virginica* (Gmelin). (Ed.) Quick Jnr, J.A. Florida Department of Natural Resources. pp. 7-35.
- BUROKER, N.E. (1982). Allozyme variation in three nonsibling *Ostrea* species. *J. Shell. Res.*, 2; pp. 157-163.
- BUROKER, N.E. (1985). Evolutionary patterns in the family Ostreidae: Larviparity vs. Oviparity. *J. Exp. Mar. Biol. Ecol.*, 90; pp. 233-247.
- BUROKER, N.E., CHANLEY, P., CRANFIELD, H.J. and DINAMINI, P. (1983). Systematic status of two oyster populations of the genus *Tiostrea* from New Zealand and Chile. *Mar. Biol.*, 77; pp. 191-200.
- BUROKER, N.E., HERSHBERGER, W.K. and CHEW, K.K. (1979a). Population genetics of the family Ostreidae. 1. Intraspecific studies of *Crassostrea gigas* and *Saccostrea commercialis*. *Mar. Biol.*, 54; pp. 157-169.
- BUROKER, N.E., HERSHBERGER, W.K. and CHEW, K.K. (1979b). Population genetics of the family Ostreidae. 2. Interspecific studies of the genera *Crassostrea* and *Saccostrea*. *Mar. Biol.*, 54; pp. 171- .
- BUXTON, C.D., NEWELL, R.C. and FIELD, J.G. (1981). Response-surface analysis of the combined effects of exposure and acclimation temperatures on filtration, oxygen consumption and scope for growth in the oyster *Ostrea edulis*. *Mar. Ecol. Prog. Ser.*, 6; pp. 73-82.
- CASTRO, E.M., URPI, O.P., MADRIZ, E.Z., QUESADA, R.Q. and MONTOYA, J.A. (1985). Filtration rates in the mangrove oyster (*Crassostrea rhizophorae*, Guilding 1828), at different salinities and temperatures. *Rev. Biol. Trop.*, 33; pp. 77-79.

- CHAGOT, D.J. (1989). *Caractérisation morphologique et fonctionnelle des hémocytes d'Ostrea edulis et Crassostrea gigas, mollusques bivalves. Études in vitro de leurs interactions avec le protozoaire Bonamia ostreae (Acetospora)*. PhD Thesis, University of Montpellier II.
- CHAGOT, D., BOULO, V., HERVIO, D., MIALHE, E., BACHERE, E., MOURTON, C. and GRIZEL, H. (1992). Interactions between *Bonamia ostreae* (Protozoa:Acetospora) and hemocytes of *Ostrea edulis* and *Crassostrea gigas* (Mollusca:Bivalvia) : Entry mechanisms. *J. Invert. Pathol.*, **59**; pp. 241-249.
- CHENG, T.C. (1976). Aspects of substrate utilization and energy requirements during molluscan phagocytosis. *J. Invert. Pathol.*, **27**; pp. 263-268.
- CHENG, T.C. (1978). *The Release of Lysosomal Hydrolases in Molluscan Cellular Response to Immunologic Challenge*. in Comparative Pathobiology, Vol. 4. (Eds) Bulla Jnr, L.A. and Cheng, T.C. Plenum Publishing Corporation. pp. 59-71.
- CHENG, T.C. (1981). *Bivalves*, in Invertebrate Blood Cells, Vol. 1. (Eds.) Ratcliffe, N.A. and Rowley, A.F. Academic Press, New York. pp. 233-300.
- CHENG, T.C. (1986). Specificity and the role of lysosomal hydrolases in molluscan inflammation. *Int. J. Tiss. Reac.*, **VIII**; pp. 439-445.
- CHENG, T.C. (1988). *In vivo* effects of heavy metals on cellular defense mechanisms of *Crassostrea virginica* : phagocytic and endocytic indices. *J. Invert. Pathol.*, **51**; pp. 215-220.
- CHENG, T.C. (1993). *Non-infectious Diseases of Marine Molluscs*, in Pathobiology of Marine and Estuarine Organisms. (Eds.) Couch, J.A. and Fournie, J.W. CRC Press. pp. 289-318.
- CHENG, T.C. and BURTON, R.W. (1965). Relationships between *Bucephalus* sp. and *Crassostrea virginica* : histopathology and sites of infection. *Chesapeake Science*, **3**; pp. 3-16.
- CHENG, T.C. and BURTON, R.W. (1966). Relationships between *Bucephalus* sp. and *Crassostrea virginica* : a histochemical study of some carbohydrates and carbohydrate complexes occurring in the host and parasite. *Parasitology*, **56**; pp. 111-122.

- CHENG, T.C. and BUTLER, M.S. (1979). Experimentally induced elevations in acid phosphatase activity in hemolymph of *Biomphalaria glabrata* (Mollusca). *J. Invert. Pathol.*, **34**; pp. 119-124.
- CHENG, T.C. and GUIDA, V.G. (1980). Behaviour of *Bulinus truncatus rohlfsi* hemocytes (Gastropoda:Pulmonata). *Trans. Amer. Micros. Soc.*, **99**; pp. 101-111.
- CHENG, T.C., GUIDA, V.G. and GERHART, P.L. (1978). Aminopeptidase and lysozyme activity levels and serum protein concentrations in *Biomphalaria glabrata* (Mollusca) challenged with bacteria. *J. Invert. Pathol.*, **32**; pp. 297-302.
- CHENG, T.C. and HOWLAND, K.H. (1979). Chemotactic attraction between hemocytes of the oyster *Crassostrea virginica*, and bacteria. *J. Invert. Pathol.*, **33**; pp. 204-210.
- CHENG, T.C. and HOWLAND, K.H. (1982). Effects of colchicine and cytochalasin B on chemotaxis of oyster (*Crassostrea virginica*) hemocytes. *J. Invert. Pathol.*, **40**; pp. 150-152.
- CHENG, T.C. and RODRICK, G.E. (1975). Lysosomal and other enzymes in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria*. *Comp. Biochem. Physiol.*, **52**; pp. 443-447.
- CHENG, T.C., RODRICK, G.E., FOLEY, D.A. and KOEHLER, S.A. (1975). Release of lysozyme from hemolymph cells of *Mercenaria mercenaria* during phagocytosis. *J. Invert. Pathol.*, **25**; pp. 261-265.
- CHENG, T.C. and SULLIVAN, J.T. (1984). Effects of heavy metals on phagocytosis by molluscan hemocytes. *Mar. Env. Res.*, **14**; pp. 305-315.
- CHEW, K.K. (1989). *Manila Clam Biology and Fishery Development in Western North America*, in *Clam Mariculture in North America*, (eds.) Manzi, J.J. and Castagna, M. Elsevier Science Publishers. pp. 243-261.
- CHOI, K.-S., WILSON, E.A., LEWIS, D.H., POWELL, E.N. and RAY, S.M. (1989). The energetic cost of *Perkinsus marinus* parasitism in oysters : quantification of the thioglycollate method. *J. Shell. Res.*, **8**; pp. 125-131.

- CHRISTIAN, M.B. (1987). *Observations of Transport in the English Channel using Lagrangian Methods*. MSc Dissertation, University of Southampton.
- CHU, F.L.E. and LAPEYRE, J.F. (1989). Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*). *J. Invert. Pathol.*, **54**; pp. 224-232.
- CHU, F.L.E. and LAPEYRE, J.F. (1993). Development of disease caused by the parasite, *Perkinsus marinus* and defense-related hemolymph factors in three populations of oysters from the Chesapeake Bay, USA. *J. Shell. Res.*, **12**; pp. 21-27.
- COMBS, M. (1983). *Recherches histologiques et cytologiques sur les infections intracellulaires des mollusques bivalves marins*. Thèse d'Etat, Université des Sciences et Techniques du Languedoc. 128 pp.
- COMPS, M., TIGÉ, G. and GRIZEL, H. (1980). Etude ultra-structurale d'un protiste parasite de l'huître plate *Ostrea edulis* L. *C. R. Acad. Sci., Paris*, **290**; pp. 383-384.
- CONOVER, R.J. (1966). Assimilation of organic matter by zooplankton. *Limnol. Oceanog.*, **11**; pp. 338-345.
- COUCH, J.A. (1985). Prospective study of infectious and non-infectious diseases in oysters and fishes in three Gulf of Mexico estuaries. *Dis. Aquat. Org.*, **1**; pp. 59-82.
- CRAWFORD, D.W., HAWKINS, L.E., HUTCHINSON, S., ANTAI, E.E., PURDIE, D.A. and LOCKWOOD, A.P.M. (1993). *Red tides of Mesodinium rubrum : evidence for remotely imposed stress on the oyster Ostrea edulis*. in Toxic Phytoplankton Blooms in the Sea. (Eds.) Smayda, T.J. and Shimizu, Y. Elsevier Science Publishers. pp. 389-394.
- CRAWFORD, D.W. and PURDIE, D.A. (1992). Evidence for avoidance of flushing from an estuary by a planktonic, phototrophic ciliate. *Mar. Ecol. Prog. Ser.*, **79**; pp. 259-265.
- CROSS, M.L., NEWKIRK, G.F. and JOHNSON, G. (1987). Strategies for the cultivation of European oysters (*Ostrea edulis*) in the Maritime provinces of Canada : preliminary analyses. *Can. J. Fish. Aquat. Sci.*, **44**; pp. 674-679.

- CUMMINS, J.M., JONES, A.C. and STEVENS, A.A. (1971). Occurrence of toxic bivalve molluscs during a *Gymnodinium breve* 'red tide'. *Trans. Amer. Fish. Soc.*, **1**; pp. 112-116.
- DIEHL, W.J., GAFFNEY, P.M. and KOEHN, R.K. (1986). Physiological and genetic aspects of growth in the mussel *Mytilus edulis*. 1. Oxygen consumption, growth, and weight loss. *Physiol. Zool.*, **59**; pp. 201-211.
- DIEHL, W.J. and KOEHN, R.K. (1985). Multiple-locus heterozygosity, mortality, and growth in a cohort of *Mytilus edulis*. *Mar. Biol.*, **88**; pp. 265-271.
- DIKKEBOOM, R., TIJNAGEL, J.M.G.H., MULDER, E.C. and VAN DER KNAAP, W.P.W. (1987). Hemocytes of the pond snail *Lymnaea stagnalis* generate reactive forms of oxygen. *J. Invert. Pathol.*, **49**; pp. 321-331.
- DINAMANI, P., HINE, P.M. and JONES, J.B. (1987). Occurrence and characteristics of the haemocyte parasite *Bonamia* sp. in the New Zealand dredge oyster *Tiostrea lutaria*. *Dis. Aquat. Org.*, **3**; pp. 37-44.
- DOERING, P.H. and OVIATT, C.A. (1986). Application of filtration rate models to field populations of bivalves: an assessment using experimental mesocosms. *Mar. Ecol. Prog. Ser.*, **31**; pp. 265-275.
- DOSSANTOS, A.E. and NASCIMENTO, I.A. (1985). Influence of gamete density, salinity and temperature on the normal embryonic development of the mangrove oyster *Crassostrea rhizophorae* Guilding 1828. *Aquaculture*, **47**; pp. 335-352.
- EBERLEE, J.C., STOREY, J.M. and STOREY, K.B. (1983). Anaerobiosis, recovery from anoxia, and the role of strombine and alanopine in the oyster *Crassostrea virginica*. *Canad. J. Zool.*, **61**; pp. 2682-2687.
- ELSTON, R.A. (1984). Prevention and management of infectious diseases in intensive mollusc husbandry. *J. World Mariculture Soc.*, **15**; pp. 284-300.
- ELSTON, R.A. (1989). *Bacteriological Methods for Diseased Shellfish*, in *Methods for the Microbiological Examination of Fish and Shellfish*, (Eds.) Austin B. and Austin D.A. Ellis Horwood Ltd. pp. 187-215.

- ELSTON, R.A., BEATTIE, J.H., FRIEDMAN, C., HEDRICK, R. and KENT, M.L. (1987a). Pathology and significance of fatal inflammatory bacteraemia in the Pacific oyster *Crassostrea gigas* Thunberg. *J. Fish Dis.*, **10**; pp. 121-132.
- ELSTON, R.A., ELLIOT, L. and COLWELL, R.R. (1982). Conchiolin infection and surface coating *Vibrio* : shell fragility, growth depression and mortalities in cultured oysters and clams *Crassostrea virginica*, *Ostrea edulis* and *Mercenaria mercenaria*. *J. Fish. Dis.*, **5**; pp. 265-284.
- ELSTON, R.A., FARLEY, C.A. and KENT, M.L. (1986). Occurrence and significance on bonamiasis in European flat oysters *Ostrea edulis* in North America. *Dis. Aquat. Org.*, **2**; pp. 49-54.
- ELSTON, R.A., KENT, M.L. and WILKINSON, M.T. (1987b). Resistance of *Ostrea edulis* to *Bonamia ostreae* infection. *Aquaculture*, **64**; pp. 237-242.
- FARLEY, C.A., WOLF, P.H. and ELSTON, R.A. (1988). A long-term study of 'microcell' disease in oysters with a description of a new genus, *Mikrocytos* (g.n.), and two new species, *Mikrocytos mackini* (sp. n.) and *Mikrocytos roughleyi* (sp. n.). *Fish. Bull.*, **86**; pp. 581-593.
- FENG, S.Y. (1988). Cellular defense mechanisms of oysters and mussels. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 153-168.
- FENG, S.Y., FENG, J.S., BURKE, C.N. and KHAIRALLAH, L.H. (1971). Light and electron microscopy of the leucocytes of *Crassostrea virginica* (Mollusca:Pelecypoda). *Zeit. Zellforsch. Mikros. Anat.*, **120**; pp. 222-245.
- FISHER, W.S. (1986). *Structure and Function of Oyster Hemocytes*, in *Immunity in Invertebrates*. (Ed.) Brehelin, M. Springer-Verlag, Berlin. pp. 25-35.
- FISHER, W.S., editor. (1988a). *Disease Processes in Marine Bivalve Molluscs*. American Fisheries Society Special Publication.
- FISHER, W.S. (1988b). Environmental influence on bivalve hemocyte function. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 225-237.

- FISHER, W.S., CHINTALA, M.M. and MOLINE, M.A. (1989). Annual variation of estuarine and oceanic oyster *Crassostrea virginica* Gmelin hemocyte capacity. *J. Exp. Mar. Biol. Ecol.*, **127**; pp. 105-120.
- FISHER, W.S., GAUTHIER, J.D. and WINSTEAD, J.T. (1992). Infection intensity of *Perkinsus marinus* disease in *Crassostrea virginica* (Gmelin, 1791) from the Gulf of Mexico maintained under different laboratory conditions. *J. Shell. Res.*, **11**; pp. 363-369.
- FISHER, W.S. and NEWELL, R.I.E. (1986). Salinity effects on the activity of granular hemocytes of American oysters *Crassostrea virginica*. *Biol. Bull.*, **170**; pp. 122-134.
- FISHER, W.S. and TAMPLIN, M. (1988). Environmental influence on activities and foreign particle binding by hemocytes of American oysters, *Crassostrea virginica*. *Canad. J. Fish. Aquat. Sci.*, **45**; pp. 1309-1315.
- FLASSCH, J.P. and LEBORGNE, Y. (1992). Introduction in Europe, from 1972 to 1980, of the Japanese Manila clam (*Tapes philippinarum*) and the effects on aquaculture production and natural settlement. *ICES Mar. Sci. Symp.*, **194**; pp. 92-96.
- FOLEY, D.A. and CHENG, T.C. (1977). Degranulation and other changes of molluscan granulocytes associated with phagocytosis. *J. Invert. Pathol.*, **29**; pp. 321-325.
- FOLTZ, D.W. and CHATRY, M. (1986). Genetic heterozygosity and growth rate in Louisiana oysters (*Crassostrea virginica*). *Aquaculture*, **57**; pp. 261-269.
- FORD, S.E. and HASKIN, H.H. (1988). Management strategies for MSX (*Haplosporidium nelsoni*) disease in Eastern oysters. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 249-256.
- FOSTER-SMITH, R.L. (1975). The effect of concentration of suspension on filtration rates and pseudofaecal production for *Mytilus edulis* (L.), *Cerastoderma edule* (L.) and *Venerupis pullastra* (Montagu). *J. Exp. Mar. Biol. Ecol.*, **17**; pp. 1-22.
- FRIEDMAN, C.S. and HEDRICK, R.P. (1991). Pacific oyster nocardiosis : isolation of the bacterium and induction of laboratory infections. *J. Invert. Pathol.*, **57**; pp. 109-120.

- FRIEDMAN, C.S., McDOWELL, T., GROFF, J.M., HOLLIBAUGH, J.T., MANZER, D. and HEDRICK, R.P. (1989). Presence of *Bonamia ostreae* among populations of the European flat oyster, *Ostrea edulis* Linné, in California, USA. *J. Shell. Res.*, **8**; pp. 133-137.
- GAFFNEY, P.M. (1986). *Physiological genetics of growth in marine bivalves*. Ph.D. dissertation. Stony Brook: State University of New York.
- GAFFNEY, P.M. and SCOTT, T.M. (1984). Genetic heterozygosity and production traits in natural and hatchery populations of bivalves. *Aquaculture*, **42**; pp. 289-302.
- GALTSOFF, P.S. (1964). *The American Oyster Crassostrea virginica (Gmelin)*. Fishery Bulletin of the Fish and Wildlife Service, volume **64**. U.S. Government Printing Office. 480 pp.
- GARTON, D.W., KOEHN, R.K. and SCOTT, T.M. (1984). Multiple-locus heterozygosity and the physiological energetics of growth in the coot clam, *Mulinia lateralis*, from a natural population. *Genetics*, **108**; pp. 445-455.
- GAUTHIER, J.D. and FISHER, W.S. (1990). Hemolymph assay for diagnosis of *Perkinsus marinus* in oysters *Crassostrea virginica* (Gmelin, 1791). *J. Shell. Res.*, **9**; pp. 367-371.
- GAUTHIER, J.D., SONIAT, T.M. and ROGERS, J.S. (1990). A parasitological survey of oysters along salinity gradients in coastal Louisiana. *J. World Aqua. Soc.*, **21**; pp. 105-115.
- GOLDBERG, E.D. (1980). *The International Mussel Watch*. National Research Council, National Academy of Sciences, Washington, D.C.
- GOULLETQUER, P., HERAL, M., DESLOUS-PAOLI, J.M., PROU, J., GARNIER, J., RAZET, D. and BOROMTHANARAT, W. (1989). Ecophysiology and energy balance of the Manila clam *Ruditapes philippinarum*. *J. Exp. Mar. Biol. Ecol.*, **132**; pp. 85-108.
- GRISCHKOWSKY, R.S. and LISTON, J. (1974). Bacterial pathogenicity in laboratory induced mortality of the Pacific oyster (*Crassostrea gigas*, Thunberg). *Proc. Natl. Shell. Assoc.*, **64**; pp. 82-91.
- GRIZEL, H., COMPS, M., BONAMI, J.R., COUSSERANS, F., DUTHOIT, J.L. and LE PENNEC, M. (1974). Recherche sur l'argent de la maladie de la glande digestive de *Ostrea edulis* Linné. *Sci. Pêche Bull. Inst. Pêches Marit.*, **240-241**; pp. 7-30.

- GRIZEL, H. and HÉRAL, M. (1990). Introduction into France of the Japanese oyster *Crassostrea gigas*. *J. Cons. Int. Explor. Mer.*, **47**; pp. 399-403.
- GRIZEL, H., MIALHE, E., CHAGOT, D., BOULO, V. and BACHERE, E. (1988). Bonamiasis : A model study of diseases in marine molluscs. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 1-4.
- HAMMEN, C.S. (1969). Metabolism of the oyster *Crassostrea virginica*. *Am. Zool.*, **9**; pp. 309-318.
- HARRIS, H. and HOPKINSON, D.A. (1976) *Handbook of Electrophoresis in Human Genetics*. (1977) supplement, (1978) supplement, Elsevier/North-Holland Biomedical Press.
- HAWKINS, A.J.B., BAYNE, B.L. and DAY, A.J. (1986). Protein turnover, physiological energetics and heterozygosity in the blue mussel, *Mytilus edulis*: The basis of variable age-specific growth. *Proc. Roy. Soc. Lond. B*, **229**; pp. 161-176.
- HAWKINS, L.E., BROOKS, J.D., BROOKS, S. and HUTCHINSON, S. (1993a). The effect of tidal exposure on aspects of metabolic and immunological activity in the hard clam *Mercenaria mercenaria*. *Comp. Biochem. Physiol.*, **104A**; pp. 225-228.
- HAWKINS, L.E. and HUTCHINSON, S. (1990). *Identification of the Stress Response in native oysters Ostrea edulis*. Ministry of Agriculture, Fisheries and Food Report CSA 1165.
- HAWKINS, L.E., HUTCHINSON, S. and BROOKS, J.D. (1993b). *Application of Immunological and Biological Water Quality Indicators in Coastal Waters*. Ministry of Agriculture, Fisheries and Food Report CSA 1801. 152 pp.
- HAWKINS, L.E., HUTCHINSON, S., BROOKS, J.D. and BUCKE, D. (1993c). *Differential Responses of Three Populations of Ostrea edulis to Environmental Stress*, in Quantified Phenotypic Responses in Morphology and Physiology, Proceedings of the 27th European Marine Biology Symposium, Dublin, Ireland, 7-11th September, 1992. (Ed.) Aldrich, J.C. JAPAGA, Ashford. pp. 207-214.
- HAZEL, J.R. and PROSSER, C.L. (1974). Molecular mechanisms of temperature compensation in poikilotherms. *Physiol. Rev.*, **54**; pp. 620-677.
- HÉRAL, M. (1986). L'ostréiculture française traditionnelle. *Aquaculture*, (Ed.) Barnabé, G. Lavoisier, Paris. p. 346.

- HÉRAL, M. and DESLOUS-PAOLI, J.M. (1993). *Oyster Culture in European Countries*. in *Estuarine and Marine Bivalve Mollusk Culture*, (Ed.) Menzel, W. CRC Press. pp. 153-190.
- HÉRAL, M., DESLOUS-PAOLI, J.M. and PROU, J. (1986). Dynamiques des productions et des biomasses des huîtres creuses cultivées (*Crassostrea angulata* and *Crassostrea gigas*) dans le bassin de Marennes-Oléron depuis un siècle. *ICES Mariculture Committee, F*, **41**; p. 22.
- HIDU, H. and LAVOIE, R.E. (1993). *The European Flat Oyster Ostrea edulis L., in Maine and Eastern Canada*, in *Estuarine and Marine Bivalve Mollusk Culture*, (Ed.) Menzel, W. CRC Press. pp. 35-46.
- HILDRETH, D.I. and CRISP, D.J. (1976). A corrected formula for calculation of filtration of bivalve molluscs in an experimental flowing system. *J. Mar. Biol. Assoc. U.K.*, **56**; pp. 111-120.
- HINE, P.M. (1991). The annual pattern of infection by *Bonamia* sp. in New Zealand flat oysters, *Tiostrea chilensis*. *Aquaculture*, **93**; pp. 241-251.
- HIS, E. (1969). Recherche d'un test permettant de comparer l'activité respiratoire des huîtres au cours de l'évolution de la maladie des branchies. *Rev. Trav. Pêches Marit.*, **33**; pp. 171-175.
- HIS, E. (1972). Premiers éléments de comparaison entre l'huître japonaise. *Sci. Pêches, Bull. Inst. Pêches Marit.*, **219**; p. 1.
- HIS, E., ROBERT, R. and DINET, A. (1989). Combined effects of temperature and salinity on fed and starved larvae of the Mediterranean mussel *Mytilus galloprovincialis* and the Japanese oyster *Crassostrea gigas*. *Mar. Biol.*, **100**; pp. 455-463.
- HUDSON, E.B. and HILL, B.J. (1991). Impact and spread of bonamiasis in the U.K. *Aquaculture*, **93**; pp. 279-285.
- HUTCHINSON, S. and HAWKINS, L.E. (1992). Quantification of the physiological responses of the European flat oyster *Ostrea edulis* L. to temperature and salinity. *J. Mollus. Stud.*, **58**; pp. 215-216.
- IMAI, T., MORI, L., SUGAWARA, Y., TAMATE, H., OIZUMI, J. and ITIKAWA, O. (1968). Studies on the mass mortality of oysters in Matsushima Bay. VII. Pathogenetic investigation. *Tohoku J. Agricult. Res.*, **19**; pp. 250-265.

- JEFFRIES, V.E. (1982). Three *Vibrio* strains pathogenic to larvae of *Crassostrea gigas* and *Ostrea edulis*. *Aquaculture*, **29**; pp. 201-226.
- JEONG, B.Y., OSHIMA, T. and KOIZUMI, C. (1991). Changes in molecular species compositions of glycerophospholipids in Japanese oyster *Crassostrea gigas* (Thunberg) during frozen storage. *Comp. Biochem. Physiol.*, **100B**; pp. 99-105.
- JOHANNESSON, K., RÖDSTRÖM, E.M. and AASE, H. (1989). Low genetic variability in Scandinavian populations of *Ostrea edulis* L. - possible causes and implications. *J. Exp. Mar. Biol. Ecol.*, **128**; pp. 177-190.
- JOHNSON, G.B. and FELDMAN, M.W. (1973). On the hypothesis that polymorphic enzyme alleles are selectively neutral. 1. The evenness of allele frequency distribution. *Theoret. Pop. Biol.*, **4**; pp. 209-221.
- JØRGENSEN, C.B. (1981). A hydromechanical principle for particle retention in *Mytilus edulis* and other ciliary suspension feeders. *Mar. Biol.*, **61**; pp. 277-282.
- JØRGENSEN, C.B. (1990). *Bivalve filter feeding: hydrodynamics, bioenergetics, physiology and ecology*. Olsen & Olsen, Denmark.
- KATKANSKY, S.C., DAHLSTROM, W.A. and WARNER, R.W. (1969). Observations on survival and growth of the European flat oyster, *Ostrea edulis*, in California. *Calif. Fish Game*, **55**; pp. 69-74.
- KENT, R.M.L. (1979). The influence of heavy infestations of *Polydora ciliata* on the flesh content of *Mytilus edulis*. *J. Mar. Biol. Assoc. U.K.*, **59**; pp. 289-297.
- KEY, D. and DAVIDSON, P.E. (1981). *A Review of Development of the Solent Oyster Fishery 1972-80*. Ministry of Agriculture, Fisheries and Food, Directorate of Fisheries Research, Lowestoft. Laboratory Leaflet No. 52. 36 pp.
- KIMURA, M. and CROW, J.F. (1964). The number of alleles that can be maintained in a finite population. *Genetics*, **49**; pp. 725-738.
- KING, M.G. (1977). Cultivation of the Pacific oyster, *Crassostrea gigas*, in a non-tidal hypersaline pond. *Aquaculture*, **11**; pp. 123-136.

- KINNE, O. (1984) *Diseases of Marine Organisms*, in *Diseases of Marine Organisms*. (Ed.) Kinne, O. and Bulnheim, H.P., *Helgol. Meeresunters.*, **37**; pp. 1-4.
- KOBAYASHI, T., ENOMOTO, S., SAKAZAKI, R. and KUWAHARA, S. (1963). A new selective isolation medium for pathogenic vibrios : TCBS agar (modified Nakanishi's agar). *Jap. J. Bacteriol.*, **18**; pp. 387-391.
- KOEHN, R.K. (1984). *The Application of Genetics to Problems in the Marine Environment : Future Areas of Research*. A discussion paper prepared for the Natural Environment Research Council. 11 pp.
- KOEHN, R.K. (1990). Heterozygosity and growth in marine bivalves: Comments on the paper by Zouros, Romero-Dorey, and Mallet (1988). *Evolution*, **44**; pp. 213-216.
- KOEHN, R.K. and BAYNE, B.L. (1989). Towards a physiological and genetical understanding of the energetics of the stress response. *Biol. J. Linn. Soc.*, **39**; pp. 157-171.
- KOEHN, R.K., BAYNE, B.L., MOORE, M.N. and SIEBENHALLER, J.F. (1980). Salinity related physiological and genetic differences between populations of *Mytilus edulis*. *Biol. J. Linn. Soc.*, **14**; pp. 319-334.
- KOEHN, R.K., DIEHL, W.J. and SCOTT, T.M. (1988). The differential contribution by individual enzymes of glycolysis and protein catabolism to the relationship between heterozygosity and growth. Rate in the coot clam, *Mulinia lateralis*. *Genetics*, **118**; pp. 121-130.
- KOEHN, R.K. and GAFFNEY, P.M. (1984). Genetic heterozygosity and growth rate in *Mytilus edulis*. *Mar. Biol.*, **82**; pp. 1-7.
- KOEHN, R.K. and SHUMWAY, S. (1982). A genetic/physiological explanation for differential growth rate among individuals of the American oyster *Crassostrea virginica* (Gmelin). *Mar. Biol. Lett.*, **3**; pp. 35-42.
- KORRINGA, P. (1941). Experiments and observations on swarming, pelagic life and setting in the European flat oyster, *Ostrea edulis* L. *Arch. Neerl. Zool.*, **5**; p. 249.
- KORRINGA, P. (1952). Recent advances in oyster biology. *Quart. Rev. Biol.*, **27**; pp. 266-308 and 339-365.

- KORRINGA, P. (1957). Water temperature and breeding throughout the geographical range of *Ostrea edulis*. *Ann. Biol.*, **33**; p. 109.
- KRISHNAMOORTHY, R.V., LAKSHMI, G.J., BIESIOT, P. and VENKATARAMIAH, A. (1980). Size related predictions of meat growth and yield in reef oysters from the Mississippi Sound. *Ind. J. Mar. Sci.*, **9**; pp. 258-262.
- LAING, I. (1993). The response of Manila clam, *Tapes philippinarum*, juveniles to nutritive stress. *J. Exp. Mar. Biol. Ecol.*, **173**; pp. 111-121.
- LAING, I. and MILLICAN, P.F. (1991). Dried algae diets and indoor nursery cultivation of Manila clam juveniles. *Aquaculture*, **95**; pp. 75-87.
- LAING, I., UTTING, S.D. and KILADA, R.W.S. (1987). Interactive effect of diet and temperature on the growth of juvenile clams. *J. Exp. Mar. Biol. Ecol.*, **113**; pp. 23-38.
- LAIRD, M. (1961). Microecological factors in oyster epizootics. *Can. J. Zool.*, **39**; pp. 449-485.
- LARSON, K.G., ROBERSON, B.S. and HETRICK, F.M. (1989). Effect of environmental pollutants on the chemiluminescence of hemocytes from the American oyster *Crassostrea virginica*. *Dis. Aquat. Org.*, **6**; pp. 131-136.
- LEGALL, G., BACHERE, E. and MIALHE, E. (1991). Chemiluminescence analysis of the activity of *Pecten maximus* hemocytes stimulated with Zymosan and host-specific Rickettsiales-like organisms. *Dis. Aquat. Org.*, **11**; pp. 181-186.
- LESSIOS, H.A. (1992). Testing electrophoretic data for agreement with Hardy-Weinberg expectations. *Mar. Biol.*, **112**; pp. 517-523.
- LEVITT, J.M. (1987). The effects of temperature and light on the VO₂ and growth rates in the Pacific oyster *Crassostrea gigas*. *Amer. Zool.*, **27**; abstract.
- LIPOVSKY, V.P. and CHEW, K.K. (1972). Mortality of Pacific oysters (*Crassostrea gigas*) : the influence of temperature and enriched seawater on oyster survival. *Proc. Natl. Shell. Assoc.*, **62**; pp. 72-82.

- LIPP, P.R., BROWN, B., LISTON, J. and CHEW, K.K. (1976). Recent findings on the summer diseases of Pacific oysters. *Proc. Natl. Shell. Assoc.*, **65**; pp. 9-10.
- LITTLEWOOD, D.T.J. (1989). Thermal tolerance and the effects of temperature on air gaping in the mangrove oyster *Crassostrea rhizophorae*. *Comp. Biochem. Physiol.*, **93A**; pp. 395-397.
- MACDONALD, B.A. and THOMPSON, R.J. (1986). Influence of temperature and food availability on the ecological energetics of the giant scallop *Placopecten magellanicus*. III. Physiological ecology, the gametogenic cycle and scope for growth. *Mar. Biol.*, **93**; pp. 37-48.
- MACKIN, J.G. (1962). Oyster disease caused by *Dermocystidium marinum* and other microorganisms in Louisiana. *Publications of the Institute of Marine Science, University of Texas*, **7**; pp. 132-229.
- MALLET, A.L., ZOUROS, E., GARTNER-KEPKAY, K.E. and FREEMAN, K.R. (1986). Genetics of growth in blue mussels: family and enzyme heterozygosity effects. *Mar. Biol.*, **92**; pp. 475-482.
- MALOUF, R.E. and BRICELJ, V.M. (1989). *Comparative Biology of Clams : Environmental Tolerances, Feeding and Growth*, in *Clam Mariculture in North America*, (eds.) Manzi, J.J. and Castagna, M. Elsevier Science Publishers. pp. 23-73.
- MANN, R. and GLOMB, S.J. (1978). The effect of temperature on growth and ammonia excretion of the Manila clam *Tapes japonica*. *Est. Coast. Mar. Sci.*, **6**; pp. 335-339.
- MARCUS, J.M., SCOTT, G.I. and HEIZER, D.D. (1989). The use of oyster shell thickness and condition index measurements as physiological indicators of no heavy metal pollution around three coastal marinas. *J. Shell. Res.*, **8**; pp. 87-94.
- MARTEIL, L. (1976). La conchyliculture française. Vol 2 : Biologie de l'huître et de la moule. *Rev. Trav. Inst. Pêches Marit.*, **40**; p. 149.
- McARDLE, J.F., McKIERNAN, F., FOLEY, H. and HUGH JONES, D. (1991). The current status of *Bonamia* disease in Ireland. *Aquaculture*, **93**; pp. 273-278.
- MCCORMICKRAY, M.G. and HOWARD, T. (1991). Morphology and mobility of oyster hemocytes - evidence for seasonal variations. *J. Invert. Pathol.*, **58**; pp. 219-230.

- McDADE, J.E. and TRIPP, M.R. (1967). Lysozyme in the hemolymph of the oyster, *Crassostrea virginica*. *J. Invert. Pathol.*, **9**; pp. 531-535.
- MEIATTINI, F. (1984). *Inorganic Peroxides*. in *Methods of Enzymatic Analysis*, Vol. VII, Metabolites 2. (Ed) Bergmeyer, H.-U. Verlag Chemie, Basle. pp. 566-571.
- MITTON, J.B. and GRANT, M.C. (1984). Associations among protein heterozygosity, growth rate, and developmental homeostasis. *Annu. Rev. Ecol. Syst.*, **15**; pp. 479-499.
- MOHANDAS, A., CHENG, T.C. and CHENG, J.B. (1985). Mechanism of lysosomal enzyme release from *Mercenaria mercenaria* granulocytes : a scanning electron microscope study. *J. Invert. Pathol.*, **46**; pp. 189-197.
- MONTES, J., ANADÓN, R. and AZEVEDO, C. (1994). A possible life cycle for *Bonamia ostreae* on the basis of electron microscopy studies. *J. Invert. Pathol.*, **63**; pp. 1-6.
- MOORE, C.A. and GELDER, S.R. (1985). Demonstration of lysosomal enzymes in hemocytes. *Trans. Amer. Micros. Soc.*, **104**; pp. 242-249.
- MORAGA, D. (1986). Polymorphisme génétique de populations cultivées de la palourde du pacifique *Tapes philippinarum*. *C.R. Acad. Sci. Paris, t. 302, Série III*, **17**.
- MORI, K., IMAI, T., TOYOSHIMA, K. and USUKI, I. (1965a). Studies on the mass mortality of the oyster in Matsushima Bay. IV. Changes in the physiological activity and the glycogen content of the oyster during the stages of sexual maturation and spawning. *Bull. Tohoku Reg. Fish. Res. Lab.*, **25**; pp. 49-63.
- MORI, K., TAMATE, H., IMAI, T. and ITIKAWA, O. (1965b). Studies on the mass mortality of the oyster in Matsushima Bay. V. Changes in the metabolism of lipids and glycogen of the oyster during the stages of sexual maturation and spawning. *Bull. Tohoku Reg. Fish. Res. Lab.*, **25**; pp. 65-88.
- MURPHY, D.J. and PIERCE, S.K. (1975). The physiological basis for changes in the freezing tolerance of intertidal molluscs. 1. Responses to sub-freezing temperatures and the influence of salinity and temperature and salinity acclimation. *J. Exp. Zool.*, **193**; pp. 313-322.

- MUSTAFA, T. and HOCHACHKA, P.W. (1971). Properties of pyruvate kinases in tissues of a marine bivalve. *J. Biol. Chem.*, **246**; pp. 3196-3203.
- NAKAMURA, M., MORI, K., INOCKA, S. and NOMURA, T. (1985). *In vitro* production of hydrogen peroxide by the amoebocytes of the scallop, *Patinopecten yessoensis* (Jay). *Dev. Comp. Immunol.*, **9**; pp. 407-417.
- NAVARRO, E. and IGLESIAS, J.I.P. (1993). *Infaunal Filter-Feeding Bivalves and the Physiological Response to Short-Term Fluctuations in Food Availability and Composition*, in *Bivalve Filter Feeders in Estuarine and Coastal Ecosystem Processes*. (Ed.) Dame, R.F. Springer-Verlag, Berlin. pp. 25-56.
- NEI, M. (1972). Genetic distance between populations. *Am. Nat.*, **106**; pp. 2
- NEWELL, C.R., SHUMWAY, S.E., CUCCI, T.L. and SELVIN, R. (1989). The effects of natural seston particle size and type on feeding rates, feeding selectivity and food resource availability for the mussel *Mytilus edulis* Linnaeus, 1758 at bottom culture sites in Maine. *J. Shell. Res.*, **8**; pp. 187-196.
- NEWELL, R.C. (1979). *Biology of Intertidal Animals*. Marine Ecological Surveys, Faversham, England.
- NEWELL, R.C., JOHNSON, L.G. and KOFOED, L.H. (1977). Adjustment of the component of energy balance in response to temperature change in *Ostrea edulis*. *Oecologia*, **30**; pp. 97-110.
- NEWELL, R.I.E. (1985). Physiological effects of the parasite MSX *Haplosporidium nelsoni* (Haskin, Stauber and Mackin) on the American oyster *Crassostrea virginica* (Gmelin). *J. Shell. Res.*, **5**; pp. 91-95.
- NEWELL, R.I.E. and BARBER, B.J. (1988). A physiological approach to the study of bivalve molluscan diseases. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 269-280.
- NIE, Z.-Q. (1993). *The Culture of Marine Bivalve Mollusks in China*, in *Estuarine and Marine Bivalve Mollusk Culture*. (Ed.) Wenzel, W. CRC Press. pp. 261-276.

- NOEL, T. (1991). *Brown Ring Disease in Tapes philippinarum : Epidemiology, Physiology and Identification*. Proceedings of the Third European Molluscan Pathology Workshop. Department of the Marine, Dublin, Ireland. 13-14 July, 1991.
- ORTON, J.H. (1928). On rhythmic periods in shell-growth in *Ostrea edulis*, with a note on fattening. *J. Mar. Biol. Assoc. U.K.*, **15**; pp. 365-427.
- PAPARO, A.A. (1989). The effect of various salinities on whole and dissected preparations of *Crassostrea virginica*. *Comp. Biochem. Physiol.*, **93A**; pp. 601-605.
- PERKINS, F.O. (1988). Structure of protistan parasites found in bivalve molluscs. *Amer. Fish. Soc. Spec. Publ.*, **18**; pp. 93-111.
- PICHOT, Y., COMPS, M., TIGÉ, G., GRIZEL, H. and RABOUIN, M.-A. (1979). Recherches sur *Bonamia ostreae* gen. n., sp. n., parasite nouveau de l'huître plate *Ostrea edulis* L. *Rev. Trav. Inst. Pêches Marit.*, **43**; pp. 131-140.
- PINGREE, R.D. (1980). *Physical Oceanography of the Celtic Sea and English Channel*. in The North-West European Shelf Seas : the Sea Bed and Sea in Motion, Volume II : Physical and Chemical Oceanography, and Physical Resources. (Eds) Banner, F.T., Collins, M.B. and Massie, K.S. Elsevier Scientific Publishing, Amsterdam. pp. 415-465.
- PIPE, R.K. (1992). Generation of reactive oxygen metabolites by the haemocytes of the mussel *Mytilus edulis*. *Dev. Comp. Immunol.*, **16**; pp. 111-122.
- POLANCO, E., MONTES, J., OUTON, M.J. and MELENDEZ, M.I. (1984). Situation pathologique du stock d'huîtres plates en Galice (Espagne) en relation avec *Bonamia ostreae*. *Haliotis*, **14**; pp. 91-95.
- PONUROVSKY, S.K. and YAKOVLEV, YU.M. (1992). The reproductive biology of the Japanese littleneck, *Tapes philippinarum* (A. Adams and Reeve, 1850)(Bivalvia : Veneridae). *J. Shell. Res.*, **11**; pp. 265-277.
- POWELL, E.N., GAUTHIER, J.D., WILSON, E.A., NELSON, A., FAY, R.R. and BROOKS, J.M. (1992). Oyster disease and climate change. Are yearly changes in *Perkinsus marinus* parasitism in oysters (*Crassostrea virginica*) controlled by climatic cycles in the Gulf of Mexico? *Mer. Ecol.*, **13**; pp. 243-270.

- PROSSER, C.L. (1973). *Comparative Animal Physiology*. Saunders, Philadelphia.
- PURDIE, D.A. and ANTAL, E.E. (1987). A seasonal study of bacterioplankton biomass and productivity in an estuary. *Proc. Third European Marine Biology Symposium*, Bangor, U.K. 14-18th September 1987.
- PURDUE, J.A. (1983). *The Relationship between the Gametogenic Cycle of the Pacific Oyster, Crassostrea gigas, and the Summer Mortality Phenomenon in Strains of Selectively Bred Oysters*. PhD Dissertation, University of Washington.
- QUAYLE, D.B. (1961). *Denman Island oyster disease and mortality, 1960*. Fisheries Research Board of Canada Manuscript Report 713, Ottawa.
- QUAYLE, D.B. (1964). Distribution of introduced marine Mollusca in British Columbia waters. *J. Fish. Res. Bd. Can.*, **21**; pp. 1155-1181.
- QUAYLE, D.B. (1969). Pacific oyster culture in British Columbia. *Can. Bull. Fish. Res. Bd.*, **169**; pp. 1-192.
- QUAYLE, D.B. (1982). Denman Island oyster disease 1960-1980. *B.C. Shell. Mari. News.*, **2**; pp. 1-5.
- READ, C.P. (1958). *Status of Behavioural and Physiological 'Resistance'*. Rice Institute Pamphlet, The Rice Institute, Houston, Texas. **45**; pp. 36-54.
- RICE, M.A. and PECHENIK, J.A. (1992). A review of the factors influencing the growth of the northern quahog, *Mercenaria mercenaria* (Linnaeus, 1758). *J. Shell Res.*, **11**; pp. 279-287.
- RODHOUSE, P.G. (1977). *Ecological Energetics of the Oyster Ostrea edulis L. in a Temperate Estuary*. Ph.D. Thesis, University of Southampton, Southampton, U.K. 167 pp.
- RODHOUSE, P.G. and GAFFNEY, P.M. (1984). Effect of heterozygosity on metabolism during starvation in the american oyster *Crassostrea virginica*. *Mar. Biol.*, **80**; pp. 179-187.
- RODHOUSE, P.G., McDONALD, J.H., NEWELL, R.I.E. and KOEHN, R.K. (1986). Gamete production, somatic growth and multiple-locus enzyme heterozygosity in *Mytilus edulis*. *Mar. Biol.*, **90**; pp. 209-214.

- RODRICK, G.E. and CHENG, T.C. (1974). Kinetic properties of lysozyme from *Crassostrea virginica* hemolymph. *J. Invert. Pathol.*, **24**; pp. 41-48.
- ROHDE, K. (1993). *Ecology of Marine Parasites*. 2nd Edition. CAB International. 298 pp.
- ROMALDE, J.L., BARJA, J.L. and TORANZO, A.E. (1990). Vibrios associated with red tides caused by *Mesodinium rubrum*. *Appl. Environ. Micro.*, **56**; pp. 3615-3619.
- ROPER, D.S., PRIDMORE, R.D., CUMMINGS, V.S. and HEWITT, J.E. (1991). Pollution related differences in the condition cycles of Pacific oysters *Crassostrea gigas* from Mankau harbour, New Zealand. *Mar. Env. Res.*, **31**; pp. 197-214.
- ROWSE, A.J. and FLETT, G.H. (1984). Effects of water temperature and salinity on elimination of *Salmonella charity* and *Escherichia coli* from Sydney rock oysters (*Crassostrea commercialis*). *Appl. Env. Micro.*, **48**; pp. 1061-1063.
- SAAVEDRA, C., ZAPATA, C., GUERRA, A. and ALVAREZ, G. (1993). Allozyme variation in European populations of the oyster *Ostrea edulis*. *Mar. Biol.*, **115**; pp. 85-95.
- SEAMAN, M.N.L. (1991). Survival and aspects of metabolism in oysters *Crassostrea gigas*, during and after prolonged air storage. *Aquaculture*, **93**; pp. 389-395.
- SHOZAWA, A. (1986). A reducing factor produced by hemocytes of *Biomphalaria glabrata* and its role in host defense. *Dev. Comp. Immunol.*, **10**; abstract, p. 636.
- SHPIGEL, M. (1989). Gametogenesis of the European flat oyster (*Ostrea edulis*) and Pacific oyster (*Crassostrea gigas*) in warm water in Israel. *Aquaculture*, **80**; pp. 343-349.
- SHUMWAY, S.E. (1977). Effect of salinity fluctuation on the osmotic pressure and Na⁺, Ca²⁺, and Mg⁺ ion concentrations in changing salinity. *Mar. Biol.*, **41**; pp. 153-177.
- SHUMWAY, S.E. and KOEHN, R.K. (1982). Oxygen consumption in the American oyster *Crassostrea virginica*. *Mar. Ecol. Prog. Ser.*, **9**; pp. 59-68.
- SINDERMAN, C.J. (1993). *Interactions of Pollutants and Disease in Marine Fish and Shellfish*, in Pathobiology of Marine and Estuarine Organisms. (Eds.) Couch, J.A. and Fournie, J.W. CRC press. pp. 451-482.

- SLOBODKIN, L.B. and RICHMAN, S. (1961). Calories/gm in species of animal. *Nature, London*, **191**; p. 299.
- SOLORZANO, L. (1969). Determination of ammonia in natural waters by the phenol-hypochlorite method. *Limnol. Oceanog.*, **14**; pp. 799-801.
- SPARKS, A.K. (1993). *Invertebrate Diseases - An Overview*, in Pathobiology of Marine and Estuarine Organisms. (Eds.) Couch, J.A. and Fournie, J.W. CRC Press. pp. 245-253.
- STEWART, J.E. (1991). Introductions as factors in diseases of fish and aquatic invertebrates. *Can. J. Fish. Aquat. Sci.*, **48**; pp. 110-117.
- TAMATE, H., NUMACHI, K., MORI, K., ITIKAWA, O. and IMAI, T. (1965). Studies on the mass mortality of the oyster in Matsushima Bay : pathological studies. *Bull. Tohoku Reg. Fish. Res. Lab.*, **25**; pp. 89-104.
- THIRIOT-QUIEVREUX, C., POGSON, G.H. and ZOUROS, E. (1992). Genetics of growth rate variation in bivalves - Aneuploidy and heterozygosity effects in a *Crassostrea gigas* family. *Genome*, **35**; pp. 39-45.
- TIGÉ, G. and GRIZEL, H. (1982) Essai de contamination d'*Ostrea edulis* Linne par *Bonamia ostreae* (Pichot *et al.*, 1979) en rivi re de Crach (Morbihan). *Rev. Trav. Inst. P ches Marit.*, **46**; pp. 307-314.
- ULANOWICZ, R.E., ALI, M.L., VIVIAN, A., HEINLE, D.R., RICHKUS, W.A. and SUMMERS, J.K. (1982). Identifying climatic factors influencing commercial fish and shellfish landings in Maryland. *Fish. Bull. U.S.*, **80**; pp. 611-619.
- UTTING, S.D. (1988). The growth and survival of hatchery-reared *Ostrea edulis* and spat in relation to environmental conditions at the ongrowing site. *Aquaculture*, **69**; pp. 27-38.
- UTTING, S.D. and SPENCER, B.E. (1991). *The Hatchery Culture of Bivalve Mollusc Larvae and Juveniles*. Laboratory Leaflet Number 68, Directorate of Fisheries Research, Lowestoft. 32 pp.
- UTTING, S.D. and SPENCER, B.E. (1992). Introductions of marine bivalve molluscs into the United Kingdom for commercial culture - case histories. *ICES Mar. Sci. Symp.*, **194**; pp. 84-91.

- VAN BANNING, P. (1982). *Some aspects on the occurrence, importance and control of the oyster pathogen Bonamia ostreae in the Dutch oyster culture.* in Proc. Int. Coll. Invert. Pathol., Brighton, (Eds.) Payne, C.C. and Burges, H.D. **3**; pp. 261-265.
- VAN BANNING, P. (1987). Further results of the *Bonamia ostreae* challenge tests in Dutch oyster culture. *Aquaculture*, **67**; pp. 191-194.
- VAN BANNING, P. (1991). *National Report on Disease Prevalence.* Proceedings of the Third European Molluscan Pathology Workshop. Department of the Marine, Dublin, Ireland. 13-14 July, 1991.
- VAN HAREN, R.J.F. and KOOIJMAN, S.A.L.M. (1993). Application of a dynamic energy budget model to *Mytilus edulis* (L.). *Neth. J. Sea Res.*, **31**; pp.119-133.
- WALLACE, D.H. (1966). Oysters in the estuarine environment. *Amer. Fish. Soc. Spec. Publ.*, **3**; pp. 68-73.
- WALNE, P.R. (1958). Growth of oysters (*Ostrea edulis* L.). *J. Mar. Biol. Assoc. U.K.*, **37**; pp. 591-602.
- WALNE, P.R. (1970). The seasonal variation of meat and glycogen content of seven populations of oysters *Ostrea edulis* L. and a review of the literature. *Fishery Investigations London, Series 2*, **26**; pp. 1-35.
- WALNE, P.R. (1979). *Culture of Bivalve Molluscs : 50 Years of Experience at Conwy*, 2nd Edition. Fishing News Books Ltd. 189 pp.
- WALNE, P.R. and SPENCER, B.E. (1971). *The introduction of the Pacific oyster (Crassostrea gigas) into the United Kingdom.* Shellfish Information Leaflet No. 2. 14 pp.
- WARD, R.D. and WARWICK, T. (1980). Genetic differentiation in the molluscan species *Littorina rudis* and *Littorina arcana* (Prosobranchia : Littorinidae). *Biol. J. Linn. Soc.*, **14**; pp. 417-428.
- WEISNER, B. (1984). *Lysozyme (Muramidase).* in Methods of Enzymatic Analysis, Vol IV, Enzymes 2. (Ed) Bergmeyer, H.-U. Verlag Chemie, Basle. pp. 189-195.

- WHITE, M.E., POWELL, E.N. and RAY, S.M. (1988a). Effect of parasitism by the pyramidellid gastropod *Boonea impressa* on the net productivity of oysters (*Crassostrea virginica*). *Est. Coast. Shelf Sci.*, **26**; pp. 359-377.
- WHITE, M.E., POWELL, E.N., RAY, S.M., WILSON, E.A. and ZASTROW, C.E. (1988b). Metabolic changes induced in oysters (*Crassostrea virginica*) by the parasitism of *Boonea impressa* (Gastropoda : Pyramidellidae). *Comp. Biochem. Physiol.*, **90A**; pp. 279-290.
- WIDDOWS, J. (1985). *Physiological Procedures*. In: The Effects of Stress and Pollution on Marine Animals, (Eds.) Bayne, B.L., Brown, D.A., Burns, K., Dixon, D.R., Ivanovici, A., Livingstone, D.R., Lowe, D.M., Moore, M.N., Stebbing, A.R.D. and Widdows, J. Praeger Press, New York. pp. 161-178.
- WIDDOWS, J. and BAYNE, B.L. (1971). Temperature acclimation of *Mytilus edulis* with reference to its energy budget. *J. Mar. Biol. Assoc. U.K.*, **51**; pp. 827-843.
- WIDDOWS, J., FIETH, P. and WORRALL, C.M. (1979). Relationships between seston, available food and feeding activity in the common mussel *Mytilus edulis*. *Mar. Biol.*, **50**; pp. 195-207.
- WIDDOWS, J., NEWELL, R.I.E. and MANN, R. (1989). Effects of hypoxia and anoxia on survival, energy metabolism, and feeding of oyster larvae (*Crassostrea virginica*, Gmelin). *Biol. Bull.*, **177**; pp. 154-166.
- WIESER, W. (1973). *Temperature Relations of Ectotherms : A Speculative View*, In: Wieser, W. (Ed.) Effects of Temperature on Ectothermic Organisms. Springer-Verlag, Heidelberg. pp. 1-23.
- WILBUR, K.M. (1983). ed. *The Mollusca*. Academic Press, New York.
- WILSON, E.A., POWELL, E.N. and RAY, S.M. (1988). The effect of the ectoparasitic pyramidellid snail, *Boonea impressa*, on the growth and health of oysters, *Crassostrea virginica*, under field conditions. *Fish. Bull.*, **86**; pp. 553-566.
- WILSON, J.H. (1987). Environmental parameters controlling growth of *Ostrea edulis* L. and *Pecten maximus* L. in suspended culture. *Aquaculture*, **64**; pp. 119-131.

- WINTER, J.E., ACEVEDO, M.A. and NAVARRO, J.M. (1984). Quempill'n estuary, an experimental oyster cultivation station in Southern Chile. Energy balance in *Ostrea chilensis*. *Mar. Ecol. Prog. Ser.*, **20**; pp. 151-164.
- YONGE, C.M. (1926). Structure and physiology of the organs of feeding and digestion in *Ostrea edulis*. *J. Mar. Biol. Assoc. U.K.*, **14**; pp. 295-386.
- YONGE, C.M. (1960). *Oysters*. Collins New Naturalist Series, London. 209 pp.
- YOSHINO, T.P. and CHENG, T.C. (1976). Experimentally induced elevation of aminopeptidase activity in hemolymph cells of the American oyster, *Crassostrea virginica*. *J. Invert. Pathol.*, **27**; pp.367-370.
- ZIMMERMAN, K.M. and PECHENIK, J.A. (1991). How do temperature and salinity affect relative rates of growth, morphological differentiation and time to metamorphic competence in larvae of the marine gastropod *Crepidula plana*. *Biol. Bull.*, **180**; pp. 372-386.
- ZOUROS, E. (1990). Heterozygosity and growth in marine bivalves: Response to Koehn's remarks. *Evolution*, **44**; pp. 216-218.
- ZOUROS, E. and FOLTZ, D.W (1984). Possible explanations of heterozygote deficiency in bivalve molluscs. *Malacologia*, **25**; pp. 583-591.
- ZOUROS, E. and FOLTZ, D.W. (1987). The use of allelic isozyme variation for the study of heterosis. *Isozymes Curr. Top. Biol. Med. Res.*, **15**; pp. 1-60.
- ZOUROS, E., ROMERO-DOREY, M. and MALLET, A.L. (1988). Heterozygosity and growth in marine bivalves: Further data and possible explanations. *Evolution*, **42**; pp. 1332-1341.
- ZOUROS, E., SINGH, S.M. and MILES, H.E. (1980). Growth rate in oysters: An overdominant phenotype and its possible explanations. *Evolution*, **34**; pp. 856-867.

APPENDICES

APPENDIX A1

ALGAL CULTURE

The dinoflagellates *Isochrysis galbana* and *Tetraselmis suecica*, and the diatoms *Phaeodactylum tricornutum* and *Chaetoceros calcitrans* were cultured according to the method of Baynes *et al.* (1979).

25 ml of nutrient enrichment solution (solution A - table A1.01) and 40 ml of sodium hypochlorite solution were added to 20 litres of chemically sterilised seawater, in an algal culture flask. For diatom culture, 10 ml of silicate solution (table A1.03) were also added. After 3-4 hours the water was neutralised using 20 ml of sterilised sodium thiosulphate (c. 200 g l⁻¹). 2 ml of vitamin stock solution (solution C - table A1.04) were then added to the algal culture flask. Approximately 1 l of previously cultured algae was added to the prepared flask, as an inoculum. Artificial lighting was provided for the flasks and approximately 5 l per minute of air was bubbled through the culture, introducing 1 - 2 % carbon dioxide.

FeCl ₃ .6H ₂ O	2.60 g
MnCl ₂ .4H ₂ O	0.72 g
H ₃ BO ₃	67.20 g
EDTA (Na salt)	90.00 g
NaH ₄ PO ₄ .2H ₂ O	40.00 g
NaNO ₃	200.00 g
Solution B (table A1.02)	2.00 ml
Distilled Water	to 2 litres.

Table A1.01 Solution A - Nutrient enrichment solution for algal culture.

ZnCl ₂	2.10 g
CoCl ₂ .6H ₂ O	2.00 g
(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	0.90 g
CuSO ₄ .5H ₂ O	2.00 g
Distilled Water	to 100 ml.

Table A1.02 Solution B - Trace metal solution for algal culture.

Va ₂ SiO ₃ .5H ₂ O	20 g
Distilled Water	to 500 ml.

Table A1.03 Silicate solution for diatom algal culture.

Vitamin B ₁₂	10 mg
Vitamin B ₁	200 mg
Distilled Water	to 200 ml.

Table A1.04 Solution C - Vitamin stock solution for algal culture.

APPENDIX A2

POLYACRYLAMIDE GEL ELECTROPHORESIS

A) Normal PAGE

The gels used were of 9% acrylamide, but a variety of different pore sizes were tested, by mixing ratios of acrylamide to bis. A ratio of 37.5 : 1 was used routinely, but gels of 29 : 1 and 19 : 1 were also used (table A2.02). The gel solution was made according to table A2.01. This solution was de-gassed to prevent oxygen inhibition of the polymerisation reaction. 25 μ l of TEMED (N,N,N',N', Tetramethylethylenediamine) and 50 mg of ammonium persulphate were added to catalyse the polymerisation.

Acrylamide stock solution	15 ml
Gel-buffer stock solution	10 ml
Distilled water	25 ml

Table A2.01 Polyacrylamide gel solution for electrophoresis.

Acrylamide ratio 37.5 : 1	
Acrylamide monomer	30.0 g
Methylene bisacrylamide	0.8 g
Distilled water	100 ml

Table A2.02 Concentrations of acrylamide used to obtain different ratios. All solutions stored at 0-5°C.

Acrylamide ratio 29 : 1	
Acrylamide monomer	34.8 g
Methylene bisacrylamide	1.2 g
Distilled water	100 ml
Acrylamide ratio 19 : 1	
Acrylamide monomer	28.5 g
Methylene bisacrylamide	1.5 g
Distilled water	100 ml

Table A2.02 *continued.*

B) SDS - PAGE

Gels were prepared in a similar manner to that described previously, using the reagents given in table A2.03. This solution was again degassed, to prevent polymerisation inhibition. The polymerisation reaction was catalysed by the addition of 25 μ l TEMED and 50 mg ammonium persulphate.

Acrylamide stock solution (37.5 : 1 ratio)	12.5 ml
1M Phosphate buffer (pH 6.8)	5.0 ml
10% (w/v) sodium dodecyl sulphate (SDS)	0.5 ml
Distilled water	32.0 ml

Table A2.03 Gel solution used for SDS-PAGE.

The lyophilised tissue samples were dissolved in a special SDS-PAGE solvent (table A2.04) before application to the gel - this gave a protein concentration of approximately 0.5 - 1.0 mg per ml.

10% (w/v) sodium dodecyl sulphate	0.5 ml
2 - mercaptoethanol	0.05 ml
Glycerol	0.5 ml
1M Phosphate buffer (pH 6.8)	0.05 ml
Distilled Water	3.9 ml

Table A2.04 Solvent for protein dissolution in SDS-PAGE.

The buffer systems used for electrophoresis are given by Harris & Hopkinson (1976). Where required the phosphate buffer system given in table A2.05 was used. Coomassie Brilliant Blue stain was used as a general protein stain for the SDS gel electrophoresis (table A2.06).

Sodium dihydrogen orthophosphate	8.05 g
diSodium hydrogen phosphate	2.60 g
Sodium chloride	0.90 g
Sodium azide	0.52 g
Distilled water	500 ml

Table A2.05 Phosphate buffer used for PAGE. This solution was adjusted to pH 6.8 with dilute HCl (0.1 mol/l) and diluted to 1000 ml with distilled water.

Coomassie Brilliant Blue 2R	0.2 % (w/v)
Distilled water	50 ml
Methanol	50 ml
Acetic acid	10 ml

Table A2.06 Coomassie blue stain used as a general protein stain in PAGE. The solution was filtered before use.

APPENDIX A3

BACTERIAL CULTURE

The original bacterial cultures were supplied by the Fish Diseases Laboratory, MAFF, Weymouth. Two strains of *Vibrio anguillarum* (2.76 and 3.72) were maintained, although it was strain 3.72 which was generally used for experimental purposes. Bacteria were cultured using both liquid and solid media, based on a generalised sea water agar (table A3.01). A *Vibrio* selective medium was also used to ensure that no other bacterial strains had contaminated the cultures.

Aquarium sea water was filtered through Whatman™ 0.1 µm cellulose nitrate filters to remove bacterial and other particulate matter; 250 ml of agar was usually used, which produced approximately 15-20 plates. Broth cultures were made as for the solid medium, without the addition of the agar. The solutions were autoclaved at 120°C for 20 minutes.

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 Sea Water	

Table A3.01 General sea water agar for bacterial culture.

The *Vibrio* selective medium used was a thiosulphate-citrate bile salt agar (Kobayashi *et al.*, 1963; table A3.02). Although the method stated that this solution does not require autoclaving, the mixture was autoclaved (at 120°C for 20 minutes) to ensure a sterile medium. The sea water was filtered as for the general media, and again 250 ml 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 Sea Water	

Table A3.02 Thiosulphate-citrate bile salt sucrose agar for selectively isolating *Vibrio* spp, as described by Kobayashi *et al.* (1963). * Tryptic soy broth, and ** Ferric chloride used in this study.

APPENDIX A4

BACTERIAL COUNTS

Bacterial broth was diluted, usually to a 1:5000 or 1:10000 concentration using filtered (through a Whatman™ 0.1 μm cellulose nitrate filter) distilled water. A solution of 4,6-diamidino-2-phenylindole (DAPI) was added at a concentration of 1 μl per ml. This mixture was incubated at room temperature for 15 minutes before filtering. This was done through two filters - the top a Millipore™ 0.2 μm black cellulose nitrate filter, and the bottom a white Millipore™ 0.2 μm 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 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 :

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

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

$A2$ = Bacteria (from filtered distilled water) per field (= 30 μm^2)

V = Volume of sample filtered

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

D = Dilution factor

100/30 = conversion factor to allow for the area counted

The following published papers were included in the bound thesis. These have not been digitised due to copyright restrictions, but the links are provided.

L.E. Hawkins, S. Hutchinson, J. Brooks, D. Bucke (1993) "Differential Response of Three Populations of *Ostrea Edulis* to Environmental Stress." Quantified Phenotypic Responses in Morphology and Physiology. Proceedings of the twenty seventh European Marine Biology Symposium. Dublin, Ireland, 7th-11th September, 1992, J.C. Aldrich ed. JAPAGA, Ashford, ISBN 0951443747.

L.E. Hawkins, J.D. Brooks, S. Brooks, S. Hutchinson (1993) "The Effect of Tidal Exposure on Aspects of Metabolic and Immunological Activity in the Hard Clam *Mercenaria Mercenaria* (Linnaeus)." Comp. Biochem. Physiol. Vol. 104a, No. 2, pp. 225-228.