DEVELOPMENT OF GENE PROBES TO P VIRUS (REOVIRIDAE) FOR DISEASE DIAGNOSIS IN CRUSTACEANS

Alison Walton

A Thesis Submitted for the Degree of PhD
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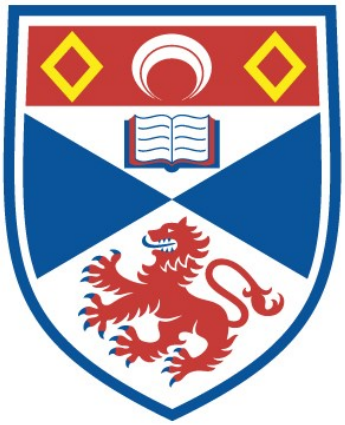
University of St Andrews



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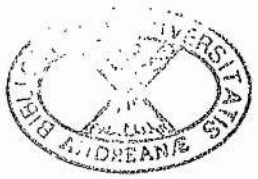
DEVELOPMENT OF GENE PROBES TO P VIRUS (REOVIRIDAE) FOR DISEASE DIAGNOSIS IN CRUSTACEANS

ALISON WALTON

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

MAY 1999



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ABSTRACT

This study reports the development of two important techniques, gene probes and haemocyte cultures, that have not been previously available to investigate viral diseases in temperate water marine decapods. These techniques were used to investigate numerous aspects of a reovirus infection of the swimming crab *Liocarcinus depurator*, P virus, both *in vivo* and *in vitro*. The construction and subsequent use of a gene probe has revealed that, not only can virus be experimentally transmitted to *L. depurator* by injection, but that it is present in natural populations of crabs from the North Sea. Seasonal variation in both incidence of P infection and in incubation time was observed. The incidence of infection increased with increasing temperature whereas incubation time decreased with increasing temperature.

In vivo, P virus was found to cause marked haemocytopenia in infected *L. depurator* and a cytopathic effect, vacuolisation of haemocytes was observed. This effect was not observed in the haemocytes of the shore crab, *Carcinus maenas*, providing evidence that P virus does not infect this species. To address the lack of techniques for *in vitro* studies, a cell culture system for crustacean haemocytes was developed. Primary culture of two haemocyte types, hyaline and semi-granular haemocytes was established for haemocytes of both *L. depurator* and *C. maenas*. High haemocyte viability was obtained for at least two weeks and, cells retained their functional capabilities *in vitro*. Having successfully established a haemocyte culture system and the gene probe E2b, it was then possible to begin investigations on P virus infections *in vitro*. P virus produced a number of effects on haemocytes of *L. depurator in vitro*. Haemocyte number and haemocyte viability decreased after addition of P virus and a number of cytopathic effects were observed such as necrosis, pycnosis and vacuolisation.

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ABBREVIATIONS

BMN	=	Baculovirus Mid-gut Necrosis Virus
BP	=	Baculovirus
CBV	=	Cheasapeake Bay Virus
CHV	=	Crab Haemopoietic Virus
ELISA	=	Enzyme-linked Immunoabsorbent Assay
FCS	=	Foetal Calf Serum
HIV	=	Human Immunodeficency
HPV	=	Hepatopancreatic Parvo-like Virus
IHHN/ IHHNV	=	Infectious and Hypodermal and Haematopoietic Necrosis Virus
MAbs	=	Monoclonal Antibodies
MBV	=	Monodon Baculovirus
NBT	=	Nitroblue Tetrazolium
PS	=	Penicillin-Streptomycin Mix (antibiotics)
RLV	=	Reo-like Virus
SOD	=	Superoxide Dismutase
TEM	=	Transmisson Electron Microscopy
THC	=	Total Haemocyte Count
TOB	=	Tetrahedral Occlusion Body
TSV	=	Taura Syndrome Virus
VSV	=	Vesticular Stomatitis Virus
WSBV	=	White Spot Baculovirus
YBV	=	Yellow Head Baculovirus

Chapter 1

GENERAL INTRODUCTION

1.1 VIRUSES IN THE AQUATIC ENVIRONMENT

It is only in the past ten years that the importance of viruses in the marine environment has been recognised. They have been found to be the most abundant component of plankton in the marine environment (Proctor, 1998). Research to date has focused on bacteriophages (Proctor *et al.*, 1988; Borsheim, 1993), although numerous other prokaryotic and eurkaryotic viruses compose the marine femtoplankton whose hosts include algae, protozoa, fungi, invertebrates, vertebrates and plants (Proctor, 1998). Free virus particles have been found in all marine and estuarine sites investigated to date, including near shore waters ranging from 0 - 50 m (Sieburth *et al.*, 1988; Bergh *et al.*, 1989; Wommack *et al.*, 1992; Heldal & Bratbak, 1991; Paul *et al.*, 1991; Cochlan *et al.*, 1993) and open ocean sites ranging from 0 - 900 m (Bergh *et al.*, 1989; Proctor & Fuhrman, 1990; Hara *et al.*, 1991; Boehme *et al.*, 1993 and Cochlan *et al.*, 1993). The abundance of viruses in the aquatic environment range from 10^4 - 10^8 per ml of sea water (Proctor, 1998). Total virus abundance seems to correlate with trophic conditions (Wienbauer & Peduzzi, 1993), bacterial abundance (Cochlan *et al.*, 1993) and chlorophyll *a* (Cochlan *et al.*, 1993). In addition, there appear to be strong seasonal and diel patterns in the abundance of bacteriophages in the marine environment (Bratbak *et al.*, 1990; Waterbury & Valois, 1993 and Jiang & Paul, 1994).

The presence of virus particles is only one of three interactive factors that determine whether a viral disease develops in the host. The concept of host, pathogen and environment as interactive factors that determine disease is demonstrated by the sphere model proposed by Snieszko (1973). In this model, spheres are used to represent variables with no constants (Fig 1.1). The host can vary, for example by species, strain, age, life cycle and nutritional status; the pathogen can vary in

virulence and the environment from ideal (as far as the host is concerned) to unsuitable. In this model, when the size of one sphere increases, for example virulence of the pathogen or perturbations in the environment, the incidence or severity of the disease increases (Snieszko, 1973).

This model, can be illustrated by examining factors contributing to disease in one group of animals, the crustaceans, which form the basis of investigations in the present study (Snieszko, 1973). The Crustacea, which belong to the phylum Arthropoda, are a mainly marine group consisting of some 26 000 species (Stewart, 1993). They are an important group with several members occupying basic positions in aquatic food chains. Indeed, some crustacean species (mainly shrimps and prawns) are being cultured extensively for human food.

In the aquatic environment, a number of natural and man-made environmental changes can affect abundance and disease in wild crustacean populations (Sindermann, 1990). Man-made changes that affect disease can include fishing and over-fishing, dredging, toxic chemicals and abnormal nutrient loads that lead to algal blooms and anoxia (Sindermann, 1990). Natural environmental changes including extreme temperatures or salinities, changes in predator balance, storms and inadequate food production affect survival and disease incidence in the wild (Sindermann 1990). However, the influence of disease, and viral diseases in particular, in wild populations of crustaceans is essentially unknown, although it is believed to have major effects on population size. Investigating viral diseases in wild populations is difficult because dead and dying crustaceans can decompose rapidly and animals weakened by disease are readily consumed by predators (Sawyer, 1991). The interactions between crustacean host, their environment and disease

causing agents and the effects of these interrelated factors on disease development are most easily studied or understood in captive or cultured populations (Lightner, 1993). The aquaculture industry provides an excellent opportunity to develop disease diagnosis and control.

1.2 THE CRUSTACEAN AQUACULTURE INDUSTRY

The husbandry of aquatic animals has been practised throughout the ages, there are unconfirmed reports of carp culture by the Chinese in the fifth century B. C. Certainly oyster culture thrived in ancient Rome and Gaul. Although most seafood is obtained directly by harvesting of fish and shellfish from untended stocks, it is recognised that wild stocks are limited and that the culture of organisms in controlled environments is becoming increasingly important. Most aquaculture today takes place to provide food for direct human consumption although other uses include production for animal feed, bait or sport. Crustacean aquaculture has a long history in Southeast Asia, where shrimp and prawn culture has been practised for at least five centuries (Bardach *et al.*, 1972). There have also been attempts to culture other crustaceans, with varying degrees of success, including freshwater crayfish, crabs and lobsters.

One of the most highly prized of all seafood is the lobster, and efforts have been made to culture it since the 1860s. Although lobsters have been successfully hatched in captivity, there are major difficulties in on-growing them under aquaculture conditions (Bardach *et al.*, 1972). In the U.S., lobster larvae are hatched from 'berried' females and maintained until the third or fourth moult at which time fry are planted out at selected points along the coast. It is difficult and, importantly, not

economically viable to raise lobsters to a marketable size from larvae in captivity (Bardach *et al.*, 1972). In the wild, lobsters take 5 - 7 years to reach marketable size, although this time can be reduced to 2 - 3 years in cultured lobsters by increasing water temperature (Bardach *et al.*, 1972). Adult lobsters must be stocked at very low densities to avoid cannibalism which has obvious drawbacks for commercial venture. At present, lobsters are ranched in Scotland (and US) which involves the capture of young adults and holding in secure cages attached to the seabed for fattening until they reach marketable size.

Efforts to culture crabs have also proved rather unsuccessful. The swimming crab, *Scylla serrata* is produced in Japan by stocking fish ponds, but, this arises because crabs enter the ponds from the wild rather than by specific attempts to culture them. The most economically important crabs are the blue crabs including primarily the species, *Callinectes sapidus* on the eastern seaboard of the United States. Attempts to culture these crabs produced large mortalities and so blue crab, and other crab industries, are almost entirely fishery operations.

Undoubtedly, the culture of penaeid shrimps has proved the most successful which has lead to a rapid growth in this industry throughout the world. Asia is the largest producer of farmed shrimps, in 1995 Asia harvested 558 000 tons of shrimp which accounts for 78 % of the world's farmed shrimp production (Hagler 1997). Thailand has been the leading Asian producer to date, producing 220 000 tons in 1995. In the western hemisphere, 154 000 tons of shrimp are produced annually of which Ecuador produces 100 000 tons. A number of species are cultured including *Penaeus orientalis, P. merguiensis, P. penicillatus, P. japonicus, P. semisulcatus* and *P. monodon* in Asia; where *P. monodon* dominates production (Fulks & Main, 1992). In the Americas almost all

of the farm raised shrimp are *P. vannamei* (Fulks & Main, 1992). There are three methods of culturing penaeid shrimps, intensive, semi-intensive and extensive systems (Lightner, 1988). The intensive system, or Galveston method (Lawrence, 1985), is predominantly used in the United States and involves stocking shrimps at high densities in intensively managed tanks or raceways (Lightner, 1988). This method can produce yields of 3 000 - 10 000 kg ha^{-1} (Lawrence, 1985). Semi-intensive systems are also used in the United States. In this system, animals are stocked at moderate densities in ponds, cages or tanks and some management is practised (Lightner, 1988), namely water exchange, feeding and fertilisation although there is usually no aeration (Lightner, 1988). The yields from semi-intensive systems are lower than those from intensive systems, 500 - 3 000 kg ha^{-1} . The extensive system, or Japanese method (Lawrence, 1985) is mainly practised in Asia using natural bodies of water. Animals are stocked at low densities with little or no management. Although water is exchanged, the animals are unfed, are not artificially fertilised and the water is not aerated. Consequently, yields from this method are lower than intensive or semi-intensive systems at 50 - 500 kg ha^{-1} (Lightner, 1988).

Disease is a major problem in intensive and semi-intensive systems where animals are stocked at high densities. For example, there are at least 30 known diseases or disease syndromes which affect cultured shrimp. They may be either infectious, communicable (viruses, bacteria, rickettsias, fungi, protozoans, helminths and parasitic crustaceans) (Stewart, 1993) or non-communicable (nutritional, physical and toxic diseases) (Lightner, 1993). Of the wide range of diseases and syndromes to affect cultured shrimp, viral diseases are perhaps the most important in penaeid shrimp culture and are known to have been responsible for

mass mortalities (Sindermann, 1990). Indeed the baculovirus, Monodon Baculovirus (MBV), was considered partially responsible for the collapse of the shrimp industry in Taiwan in the late 1980s (Lin, 1989). The picornavirus, Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV) has been reported to cause mortalities of 80-90 % in juvenile *P. monodon* (Lightner *et al.*, 1983). To date, although various treatments have been tried, they are not effective in large scale culture systems (see 1.5) so prevention is the best control measure. The need to develop techniques for rapid diagnosis and control of disease has been recognised and is the focus of much research at the present time.

1.3 VIRAL DISEASES OF CRUSTACEA

Vago (1966) discovered the first marine virus disease in a crustacean, in the swimming crab, *Liocarcinus (Macropipus) depurator* (Vago, 1966). Subsequently, several viruses have been described (see reviews by Johnson, 1983, 1984; Vega-villasante & Puente, 1993). The virology of Crustacea is a field which is comparatively recent and not very well advanced, only a few crustacean viruses have been characterised to the extent that they can be classified as a particular virus family (Bonami & Lightner, 1991). Those which can not be assigned to a virus family with certainty are tentatively related to a family on the basis of morphological and developmental characteristics as well as location in the cell (Johnson, 1984). A summary of some important viruses, found in Crustacea, and their characteristics is presented in Table 1.1 and Fig. 1.2. An outline of the important virus groups infecting crustaceans from the genus Penaeidae, Carcinidae and Portunidae, their major characteristics, symptoms and pathology is presented below. A more

detailed account of each disease can be found in the reviews of Johnson, 1983; 1984; Bonami & Lightner, 1991 and Vega-villasante & Puente, 1993).

1.3.1 DNA VIRUSES

BACULOVIRIDAE

This virus family consists of rod-shaped (bacilliform), enveloped virions which have a circular double stranded DNA genome (Dimmock & Primrose, 1987). Baculoviruses are 40 - 60 nm x 200 - 400 nm with an outer membrane and can be occluded in a protein inclusion body containing one particle (granulosis viruses) or many particles (polyhedrosis viruses) (Dimmock & Primrose, 1987) (Fig. 1.2). They infect penaeid shrimps (Johnson & Lightner, 1988; Johnson, 1988b; Vega-villasante & Puente, 1993) as well as carcinid (Bazin *et al.*, 1974; Pappalardo & Bonami, 1979 and Mari & Bonami, 1986) and portunid crabs (Johnson, 1976; 1978; 1983) (Table 1.1). In some baculoviruses, for example BP, no particular signs or symptoms have been associated with disease (Vega-villasante & Puente, 1993); although generally, infected animals exhibit poor growth, anorexia and lethargy. Baculoviruses infect hepatopancreatic and mid-gut epithelial cells in penaeids (Shariff & Subasinghe, 1992) but connective tissues and haemocytes in portunids and carcinids (Johnson, 1976; 1978; 1983; Mari & Bonami, 1986). Pathological signs of infection include nuclear hypertrophy, nucleolar degeneration (Fukuda *et al.*, 1988), necrosis, sepsis and haemocyte encapsulation (Lightner & Redman, 1981). These viruses have been responsible for major economic losses in the shrimp industry, notably MBV (see Table 1.1), which has caused heavy mortalities in *P. monodon* (Shariff & Subasinghe, 1992).

PARVOVIRIDAE

Viruses in the family Parvoviridae are icosohedral in shape with a single-stranded DNA genome and range in size from 18 - 26 nm (Dimmock & Primrose, 1987) (Fig. 1.2). Two viruses have been tentatively assigned to this family, HPV (Hepatopancreatic Parvo-like Virus), which infects penaeids (Lightner & Redman, 1985; Lightner, 1985; Lightner *et al.*, 1985; 1989 Paynter, *et al.*, 1985) and PC 84 which infects *Carcinus mediterraneus* (Mari & Bonami, 1988) (see Table 1.1). Symptoms of infection by parvoviruses include poor growth and anorexia (Bonami & Lightner, 1991); and in HPV infection of penaeids, also reduced preening and opacity of abdominal muscles (Lightner & Redman, 1985). Parvoviruses have part of their replication stage in the nucleus of infected cells (Dimmock and Primrose, 1987). Indeed, the nuclei of infected cells from penaeids and carcinid crabs can contain intranuclear inclusions of virus (Lightner & Redman 1985; Mari & Bonami 1988a). In cultured *Penaeus merguiensis* and *P. semisulcatus*, presence of HPV has been associated with high accumulative mortalities (Lightner & Redman 1985).

1.3.2 RNA VIRUSES

REOVIRIDAE

The family, reoviridae, are icosohedral in shape with a double-stranded RNA genome, consisting of 10 - 12 segments, and range in size from 60 - 80 nm (Fig. 1.2). Reoviruses, or reo-like viruses are found in penaeid shrimps (Lighnter *et al.*, 1985; Anderson *et al.*, 1987; Tsing & Bonami, 1987; Nash *et al.* , 1988; Krol *et al.*, 1990), Portunid crabs (Vago, 1966; Bonami, 1973; 1980; Johnson & Bodammer, 1975; Johnson, 1977) and Carcinid crabs (Mari & Bonami, 1988b; Mari, 1987) (see Table 1.1).

Symptoms of reovirus infection are similar to those of other viral infections, namely loss of appetite, lethargy and lack of mobility. Progressive paralysis has been observed in reoviral infections of *L. depurator* (Bonami 1973), and *C. sapidus* (Johnson 1983). At the ultrastructural level, reovirus are often found in membrane bound cytoplasmic inclusion bodies through the cytoplasm in penaeid shrimps (Lightner, *et al.*, 1985). These inclusion bodies form paracrystalline arrays in portunid crabs (Johnson, 1984; Bonami *et al.*, 1976) and rosette arrays in carcinid crabs (Mari, 1987; Mari & Bonami, 1988b). Reoviruses are particularly interesting because they are often found in association with other viruses for example, bunyavirus virus (S virus) in *L. depurator* (Bonami 1973) baculoviruses in *P. monodon* (Nash *et al.*, 1988) and rhabdoviruses in *C. sapidus* (Johnson 1984). Although high mortality levels have been recorded in animals infected with reovirus or reovirus-like agents (Johnson, 1978; Bonami, 1980; Nash *et al.*, 1988), it is difficult to determine if reovirus is directly responsible for death, acts synergistically with other pathogens or is present as endemic sub clinical infections.

Reoviruses that infect vertebrates have been extensively studied for many years (reviewed by Tyler & Fields, 1996; Virgin *et al.*, 1997). They have proved to be excellent models in vertebrate systems providing important information on how viruses cause disease (pathogenesis), the mechanisms underlying the severity of disease (virulence) and the basis for associations between infection and damage to specific cells or tissues (tropism). The extensive background knowledge of stages of reovirus pathogenesis in animals and the steps in reovirus infection of cells has also facilitated analysis of how the immune system protects the host (Virgin *et al.*, 1997). For invertebrates, nothing is known about host-virus

interactions at the cellular level, and hence it is difficult to determine pathogenesis, virulence and tropism of this virus in crustacean tissues. However, given that the virus has been physically and biochemically characterised (Montanie 1992; Montanie *et al.*, 1993), and that this group is already well established as a vertebrate model, it may prove a useful tool in crustacean virology. Due to the importance of reovirus and its potential as a model to investigate immunity to viruses in crustaceans, a virus from this family is investigated in the present study.

BUNYAVIRIDAE

These viruses are pleomorphic in shape but are most commonly observed as spheres or ovoids (Dimmock & Primrose, 1987). Virions are enveloped, approximately 100 nm in size and the genome consists of three segments of single-stranded RNA (Dimmock & Primrose, 1987) (Fig. 1.2). To date, bunyaviruses have only been found in *Carcinus maenas, Carcinus mediterraneus* and *Liocarcinus depurator* (see Table 1.1). Symptoms of Crab Haemopoietic Virus (CHV) in *C. maenas* include abnormal clotting of haemolymph (Bang, 1971) and in S virus infection of *L. depurator* include anorexia, reduced activity and progressive weakness leading to death of infected animals (Bonami & Lightner, 1991). Both viruses are found in cytoplasmic vesicles associated with the Golgi apparatus of haemocytes (Bang, 1971; Bonami, 1977; Hoover & Bang, 1978). S virus is often found in association with P virus, a reoviral infection of *L. depurator* (Bonami, 1980). There is no conclusive data on mortality levels of this infection, other than in *L. depurator* experimentally inoculated with S virus, when high levels of mortality were reported (Bonami & Lightner, 1991).

PICORNAVIRIDAE

Picornaviruses are icosohedral in shape with a single-stranded RNA genome and range in size from 20 - 30 nm in size (Dimmock & Primrose, 1987) (Fig. 1.2). Picornaviruses have been found in penaeid shrimps (Vega-villasante & Puente, 1993) and portunid crabs (Johnson, 1978; 1983; 1984; Bonami, 1980) (see Table 1.1). IHHNV (Infectious Hypodermal and Haematopoietic Necrosis Virus), which has been tentatively assigned to the picornavirus group, infects a number of species of penaeid shrimps. IHHNV infection has many symptoms in common with other viral infections, such as lethargy and loss of appetite, although in acutely affected individuals, the abdominal musculature can appear opaque and the cuticular epidermis develops several melanised areas (Lightner *et al.*, 1983). These symptoms are also reported for CBV (Cheasapeake Bay Virus) infection of *C. sapidus*, in addition, the moult pattern in this species is disrupted and animals often become blind (Johnson, 1978). The histopathology of acute and sub-acute IHHNV disease is dominated by the presence of large intranuclear, Type A inclusion bodies in hypertrophied nuclei of ectodermally derived tissues, such as striated muscle, heart, gonads, haemocytes and haematopoietic tissues (Lightner *et al.*, 1983). Such inclusion bodies are also observed in *C. sapidus*. Picornavirus infections are responsible for major losses in both penaeids and portunids. In intensive aquaculture systems, in particular, IHHNV produces mortality of up to 90 % in juveniles of *Penaeus stylirostris* (Lightner *et al.*, 1983).

1.4 MANAGEMENT AND CONTROL OF DISEASES IN THE CRUSTACEAN AQUACULTURE INDUSTRY

1.4.1 DISEASE PREVENTION

There are numerous non-pathogenic and pathogenic factors which can contribute to the development of disease in aquaculture systems (Fulks & Main, 1992). Non-pathogenic factors include:

- use of high temperatures to accelerate larval growth
- deterioration of ponds
- high stocking densities
- poor artificial feeds
- indiscriminate use of medicines and antibiotics
- use of polluted water (either inadvertently or unavoidably)
- lack of technical training for farmers
- absence of reliable sanitation

Pathogenic factors include:

- viruses
- bacteria
- bacterial and protozoan epicommensal infestations

To prevent viral diseases it is essential to control the above factors by adopting good culture practises and quarantine procedures; stock specific pathogen free (SPF) shrimps and vaccinate stock (Fulks & Main, 1992). Good culture practises are disinfecting tanks, drying ponds between cycles, optimising feeding regimes and using high quality feeds (Fulks & Main, 1992). Even when such preventive measures are adopted there is

still possibility of a disease outbreak, therefore, it is essential to be able to diagnose diseases rapidly and effectively (Fulks & Main, 1992).

1.4.2 DISEASE DIAGNOSIS

MICROSCOPY

The most widespread tool in disease diagnosis is microscopy. Light microscopy is useful to detect signs of viral infection in tissues, for example inclusion bodies (Fulks & Main, 1992). The baculovirus (BP), is readily diagnosed in either wet mounts or histological preparations of mid-gut and hepatopancreas by the presence of occlusion bodies (Lightner *et al.*, 1985). In another baculovirus infection, MBV, malachite green stain (1 % aqueous solution) in wet mounts is used to help identify spherical occlusions and to distinguish them from lipid droplets and secretory granules (Lightner, 1985). Electron microscopy can be important in some applications and is the only certain method to diagnose reovirus infections (Lightner, 1992). The technique is, however, technically more difficult and time consuming. Although microscopy is used extensively in disease diagnosis there are a number of disadvantages. Histological examination of tissue is usually performed on tissue from dead animals, although Bell *et al.*, (1990) managed to develop a procedure for the non-destructive detection of IHHNV. This technique involves excision of the first pereiopod (walking leg), followed by standard histological examination of the nerve cord of the appendage for the presence of Cowdry type A, intranuclear inclusion bodies (Bell *et al.*, 1990). Microscopy is also time consuming and, perhaps most importantly, it is not sensitive enough to detect latent infections (Fulks & Main, 1992).

ENHANCEMENT AND BIOASSAYS

In order to detect asymptomatic viral infections, enhancement and bioassay techniques have been utilised, notably for IHHNV infections (Lightner *et al.*, 1992). The purpose of enhancement is to increase the prevalence and/or severity of infection within a captive population to increase the chances of a positive diagnosis in populations from which direct sampling might give a negative diagnosis (Lightner *et al.*, 1992). In the application of this technique for IHHNV, a population of post larval shrimp were reared under relatively crowded and stressful conditions and samples taken for histopathological examination at 30 - 60 days (Lightner *et al.*, 1992). If IHHNV is present its prevalence or severity should be found to be increased to diagnosable levels (Lightner *et al.*, 1992). However, this technique has limited use as a diagnostic method because it is expensive and it is increasingly difficult to obtain pathogen free stock.

Asymptomatic infections by IHHNV can also be diagnosed using a bioassay based method (Lightner *et al.*, 1983; 1987; Lightner & Redman, 1992). The bioassay procedure involves feeding a specific pathogen free "indicator" population of shrimps with a suspect or "test" shrimp over a fourteen day period (Lightner *et al.*, 1992). If IHHNV is present in the "test" shrimp, the "indicator" shrimp typically should display diagnosable IHHN disease in histopathological samples on or after 14 days of a 28 day bioassay (Lightner & Redman, 1992). The disadvantages of the bioassay method are that microscopical examination is essential, with its associated limitations (see above), and it is extremely laborious and expensive to maintain isolation laboratories. It is also becoming increasingly difficult to find SPF stocks of shrimp.

1.4.3 RECENT ADVANCES IN DIAGNOSTIC PROCEDURES

In recent years, new diagnostic methods are beginning to be developed for viral diseases. The criteria for such techniques are that they must be rapid, simple, inexpensive, easily standardised and more reliable than existing techniques (Lightner *et al.*, 1992). In human and veterinary medicine, techniques such as cell and tissue culture, gene probes and serological methods have become widespread (reviewed by Teo, 1990; Freshney, 1983; Paul & White, 1973). The current status of developments in techniques for penaeid shrimps and other decapods is presented in Table 1.2. Greatest progress has been made in the development of gene probes, which are now recognised as the tool with the best available diagnostic sensitivity (Lightner *et al.*, 1992). A few are now produced commercially (Carr *et al.*, 1996). Other technologies, particularly cell and tissue culture, have yet to be successfully developed for routine use in crustacean virology. Technology to date in this field is limited to development of some primary cultures (Chen *et al.*, 1986; Ellender *et al.*, 1992).

SEROLOGY

Monoclonal antibodies have been produced for IHHNV and used successfully to distinguish between IHHNV-infected and uninfected shrimp of known disease status in archived samples (samples stored at -70° C for more than one year) (Poulos *et al.*, 1994a). However, all six monoclonal antibodies (MAbs) to IHHNV produced variable results in fresh tissue of known IHHNV status from history, histopathology or bioassay (Poulos *et al.*, 1994a). Poulos *et al.*, (1994a) determined that fresh clinical specimens elicit a non-specific response of variable intensity in indirect ELISA. Therefore, until a method of blocking non-specific

signals without damaging viral epitopes can be developed, use of MAbs in disease diagnosis is limited.

GENE PROBES AS DIAGNOSTIC TOOLS

The use of probes specific to the particular viral genome of interest is becoming increasingly important in aquaculture to detect the presence of a viral infection early enough to control its outbreak. The first gene probes developed for viral diseases in penaeid shrimps were developed in 1993, for IHHN (Mari *et al.*, 1993) and for BP (Bruce *et al.*, 1993; 1994). Gene probes for IHHN were constructed by labelling a number of cDNA fragments, whose restriction maps had been determined, with the DNA label digoxygenin-11-dUTP (Mari *et al.*, 1993). An ELISA-based system is used for the final detection (Mari *et al.*, 1993). The fragments tested for suitability as gene probes were the complete insert (BS4.5), removed by digestion with Sac I and Bam HI; and three fragments obtained by a double digestion with Eco RI and Bam HI (1.0, 2.3, and 1.3kbp). The full insert hybridised with all three bands of the viral genome confirming that at least part of the cloned DNA corresponds to the viral genome (Mari *et al.*, 1993). There was no hybridisation between two of the fragments at the extremities (1.0 and 1.3) but they hybridised with the central part, indicating homologous sequences expected from analysis of the restriction maps. The specificity of the probe BS4.5 was determined by dot blotting using 10-fold dilutions of extracted IHHNV DNA, purified IHHN virions, homogenised tissues from known IHHNV-infected animals, healthy animals, and purified hepatopancreatic parvo-like virus (Mari *et al.*, 1993). The results clearly indicated a good degree of specificity, except for some cross-reaction with the pUC 18 plasmid (due to slight contamination of the insert by some pUC18 fragment residues).

Attempts to test the probe with four different insect parvoviruses were unsuccessful, underlining the high specificity of the probe (Mari *et al.*, 1993). *In situ* hybridisation on paraffin embedded sections of healthy and infected crabs revealed no reaction with healthy tissues and, importantly, low levels of infection in some sections were easily noted, even when no histological signs were apparent. These results were the first successful cloning of a viral pathogen of a marine invertebrate and the high specificity of the probe have resulted in these probes being routinely used for disease diagnosis in Lightner's laboratory at the University of Arizona (Mari *et al.*, 1993).

Bruce *et al.*, 1993 developed gene probes for BP. These probes, labelled with digoxigenin were used for *in situ* hybridisation and all displayed a positive reaction to BP infected tissue although to varying extents, probe B1.23 (1.23kbp) gave the most intense reaction. No reaction was observed in BP-negative tissues (Bruce *et al.*, 1993). BP infections are characterised by the presence of tetrahedral occlusion bodies (TOBs) observed microscopically in squash preparations of hepatopancreas, midgut or faeces, or in histological sections (Couch, 1974; Bruce *et al.*, 1993). The TOBs frequently did not exhibit a reaction with the probe, usually when they had erupted from the cells and were in the lumen of the gut. However, the purple precipitate was sometimes found in cells which had contained TOBs or even on the outside of the TOBs themselves. Intense reactions also occurred in infected cells that did not contain TOBs which were presumably in the early stages of infection (Bruce *et al.*, 1993). Some of the probes were more effective than others and since the intensity of the reaction did not relate to the size of the probe, either directly or inversely, it is thought that the affinity of a probe for target nucleic acid depends largely on its internal sequence. There was

also a variation in the intensity of the probe between tissues, which appeared to be independent of geographical origin of the host, species or level of infection. A possible factor suggested is a difference in the stage of viral replication, which would influence the amount of viral DNA and mRNA available as target for the probe (Bruce *et al.*, 1993). The probe precipitate is only formed in epithelial cells of the midgut and hepatopancreas, its absence in other tissues supports the hypothesis that BP and other penaeid baculoviruses infect enteric tissues only. There was also no reaction in the reproductive system which strongly suggests that the virus is transmitted horizontally by the *per os* route rather than by true vertical transmission routes (Bruce *et al.*, 1994).

Since the development of the first probes for shrimp viruses (Bruce *et al.*, 1993; Mari *et al.*, 1993), probes have been constructed and used successfully to detect several crustacean viral diseases including MBV (Mari *et al.*, 1993; Poulos *et al.*, 1994b); HPV (Mari *et al.*, 1995); WSBV (Durand *et al.*, 1996) and TSV (Taura Syndrome Virus) (Mari *et al.*, 1998). It is important to emphasis that although gene probes are undoubtedly the most sensitive diagnostic tools developed to date, for example, the IHHN probe has been estimated to detect IHHNV DNA at the 0.1 pg level (Mari *et al.*, 1993), they are not infallible. The construction and development of gene probes requires rigorous testing at every stage and the hybridisation procedure numerous controls to eliminate the possibility of false negative or positive signals. Improvements have been made in the construction of gene probes, many are now produced using PCR which allows large quantities of probe to be produced reliably and rapidly (Lu *et al.*, 1993; Nunan & Lightner 1997). The construction of probes by PCR may allow the development of *in situ* PCR to detect viruses. This new technique has recently been used in human and

veterinary medicine (Walker *et al.*, 1995). *In situ* PCR combines the sensitivity of PCR reaction to intracellular localisation of genomic sequences and has the same specificity as *in situ* hybridisation (Walker *et al.*, 1995). Target nucleic acid sequences can be amplified by PCR then detected by *in situ* hybridisation (Walker *et al.*, 1995). Obviously, such technology could have important implication in diagnosis of viral diseases in crustaceans, particularly in diagnosing latent infections.

CELL AND TISSUE CULTURE TECHNOLOGY

Mammalian cell and tissue culture techniques have been available since the turn of the century (Harrison, 1907; Carrel, 1912). They are now used routinely to study a variety of cytological phenomena, for example, the cell cycle, leucocyte maturation, virus replication and cellular immune processes. By contrast, the culture of cells from lower vertebrates and invertebrates has been largely ignored, and few of these research milestones have been applied to the culture of non-mammalian cells *in vitro*. Accordingly, there is a notable dearth of cell lines from aquatic or marine animals, a situation of considerable importance in aquaculture where there is a great need for established cell lines from commercially important species to expedite disease diagnosis. The first leukocyte cell line from a teleost was not developed until 1979 when Ellender *et al.* (1979) produced one from the spleen of the silver perch, *Bairdiella chrysura*. Subsequently, cell lines, mainly from peripheral blood, spleen or kidney, have been established from carp, *Cyprinus carpio* (Faisal & Ahne 1990), channel catfish, *Ictalurus punctatus* (Lin *et al.*, 1992), spot, *Leiostomus xanthurus* (Sami *et al.*, 1992), black porgy, *Acanthopagrus schlegeli* (Tung *et al.*, 1991) and Japanese eel, *Anguilla japonica* (Chen *et al.*, 1982).

With respect to invertebrates, despite the huge diversity of invertebrate species and their enormous potential as *in vitro* models for biomedicine and ecotoxicology as well as in shellfish production, there are relatively few reports of cell culture methodologies. The first invertebrate tissue cell lines were produced for insects (Grace, 1962), arachnids, crustaceans and molluscs (Vago & Quiot, 1969). Since these pioneering studies, there have been a number of attempts to develop cell culture techniques for groups including sponges (Pomponi *et al.*, 1997), molluscs (Domart-Coulon *et al.*, 1994; Wen *et al.*, 1993; Mortensen & Glette, 1996), shrimps (Ellender *et al.*, 1992; Lu *et al.*, 1995; Loh *et al.*, 1997) and ascidians (Raftos *et al.*, 1990; Rinkevitch & Rabinowitz, 1993; Peddie *et al.*, 1995). Most species have received attention only because their cells or tissues produce metabolites of possible pharmacological significance (Pomponi *et al.*, 1997) or because the host serves as a vector for insect pathogens (Gong *et al.*, 1997). There seems to have been little attempt to culture marine invertebrate cells for fundamental studies of cell function, cytopathology or pathogen propagation. Moreover, most reports are for primary culture; immortal cell lines appear to be particularly difficult with invertebrates because of problems associated with authentication and mitotic stimulation (Freshney, 1983; Pomponi *et al.*, 1997). Indeed, some reports of cell lines from marine invertebrates have actually turned out to have been fungal or protozoan contaminants (Pomponi *et al.*, 1997).

As far as crustaceans are concerned, recent attempts to establish a culture system for cells derived from tissues were for *P. monodon* (Chen *et al.*, 1986). Although no cell outgrowth was observed in cultures of fragments of gill, hepatopancreas, nerve, muscle or gut, observable growth was obtained in the culture of gonad and heart cells (Chen *et al.*,

1986). Gonad cells were maintained using a modified Liebovitz 15 medium for 7 - 10 days at 28 -31°C during which time a confluent monolayer formed (Chen *et al.*, 1986). Primary culture of the epithelial cells could be maintained for approximately 2 weeks (Chen *et al.*, 1986). Since this study, there have been few other attempts to culture crustacean cells (Ellender *et al.*, 1992; Lu *et al.*, 1995; Loh *et al.*, 1997). Interestingly, very little research has focused on haemocyte cell culture, these cells play an essential role in host defence (Bauchau, 1981; Smith & Söderhäll, 1986; Söderhäll & Cerenius, 1992) and thus to some degree, susceptibility to viral infections (Mari 1987; Bonami 1980; Bazin *et al.*, 1974; Mari & Bonami, 1986; Johnson, 1976; 1978; 1983; Johnson & Bodammer, 1975).

There has been only one previous report of successful primary culture of haemocytes from decapods (Ellender *et al.*, 1992). In this study, circulating cells from the shrimps, *P. vannemi* or *P. aztectus* were maintained for 3 - 4 weeks *in vitro* (Ellender *et al.*, 1992). Unfortunately the authors provided no information about the viability or functionality of the cells over this period. No doubt there are many reasons why crustacean haemocytes are difficult to maintain under prolonged culture conditions. There may be, for example, a number of distinct cell populations which have varying physiological requirements *in vitro*. Furthermore, they rarely exhibit proliferation *in vitro* or *in vivo*, and they are highly sensitive to non-self materials, frequently undergoing degranulation, clotting or cell aggregation upon exposure to trace amounts of bacterial endotoxin (Smith & Söderhäll, 1986). Clearly, cell cultures are an important tool in diagnosis of virus diseases and require further development for future research on viral pathogenicity.

IN VITRO PROPAGATION OF VIRUS

Cell culture has been used since the 1950s in human and veterinary virology. The first successful use of cell culture for virus propagation was in 1949 when Enders *et al.*, cultured the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues (Enders *et al.*, 1949). Since this early application, cell cultures have become an essential tool in virology where they are used for a wide range of applications which include the production of primary isolates of natural wild-type strains of many, but not all viruses (Paul, 1971). They have been used for measuring infectivity using the quantal (end point) or particle (plaque) assay or using plaque assays to obtain genetically pure populations (Dulbecco, 1952); for assaying of sera and body fluids for presence of neutralising antibodies (Paul & White, 1973); demonstrating the transforming capacity of tumour viruses *in vitro* (Gey *et al.*, 1952) or obtaining large quantities of purified virus, an essential prerequisite for many biochemical, structural and molecular studies (Nathanson, 1997).

To date, there are only two reports of the use of cell and tissue culture technology to investigate viral pathogens of crustaceans (Chen & Kou 1989; Lu *et al.*, 1995). Chen & Kou (1989) reported infection of primary cell cultures, prepared from the lymphoid (Oka) organ of *P. monodon*, with MBV. Localised cytopathic effects were observed at 2 - 3 days after incubation of cell cultures with MBV, and viral infection was confirmed by inoculation of media from infected cultures into new monolayers (Chen & Kou 1989). The presence of MBV was also confirmed by TEM (Chen & Kou 1989). In 1995, the first quantal assay for Yellow Head Baculovirus, YBV, of penaeid shrimps in primary cell culture was developed (Lu *et al.*, 1995). Primary lymphoid organ cells of penaeid shrimps were grown in 96-well tissue culture plates and

inoculated with a serial dilution of 15 % filtered gill suspension (Lu *et al.*, 1995). Cytopathic effects were used to confirm viral infection and an infectious titre of virus was determined from this assay (Lu *et al.*, 1995). The lack of suitable cell culture technologies is undoubtedly preventing the development of *in vitro* assay systems for crustacean viruses. It is essential to study crustacean viruses, not only to facilitate disease diagnosis, but also to enhance our understanding of immune responses to viral infection in the continuing search for antiviral agents applicable to crustaceans.

1.5 DISEASE TREATMENT

In aquaculture systems, attempts to control outbreak of viral diseases begin with preventative measures (described in section 1.4.1). However, when preventative measures fail, treatments need to be applied to control the spread of infection. The treatment of disease can vary from chemotherapy (using chemicals or antibiotics) to the eradication of infected stocks and disinfection of all equipment, depending upon the identity of the pathogen and the severity of the disease (Bower, 1988). However, it must be stressed that, to date, there are no effective therapeutic or prophylactic counter measures to combat viral diseases in the penaeid culture industry (Itami *et al.*, 1998).

A number of treatments are currently being used in Asian countries to combat disease including saponin, formalin, malachite green, Treflan® , chloramphenicol, oxytetracycline and furanace (Fulks & Main, 1992). The use of such treatments is mostly applied to hatcheries, where dense groups of animals are present in small volumes of water (Fulks & Main, 1992). It is not cost-effective to combat diseases

encountered in extensive or semi-intensive systems because of the large quantities of drugs or antibiotics required. In the United States, Cutrine-Plus® is approved for use as an algicide and formalin is used to combat epicommensal protozoans (Fulks & Main, 1992). Recently, Maeda *et al.*, (1998), found that some Baculoviruses responsible for White Spot Syndrome (WSS) could be inactivated by the disinfectants, sodium hypochlorite (at maximum 10 ppm) or povidone-iodine (at maximum 25 ppm). they could also be inactivated by heat (> 50° C), drying (> 30° C) or high salt concentrations (Maeda *et al.*, 1998). However, it is unlikely that these concentrations would inactivate viruses in ponds because organic materials, such as residual feed and faeces reduce the virucidal effect of these chemicals (Maeda *et al.*, 1998). In addition, Meyer (1991) suggested that chemotherapy should only be used as an emergency, 'last resort', measure because whilst chemical treatments can reduce the incidence of disease they can also have negative effects on pond biota and flora of biological filters (Meyer 1991). There is also the danger that some chemicals can be hazardous to the user or leave undesirable, harmful residues in cultured animals (Meyer 1991). Clearly, there is a need to develop safe and effective treatments to combat viral disease. If viral diseases are to be controlled effectively research needs to focus on understanding more about viral immunology, viral pathogenesis and virus - cell interactions.

1.6 ANTIVIRAL IMMUNITY

1.6.1 INNATE IMMUNITY TO VIRUSES IN VERTEBRATES

In vertebrates, the first line of defence against viral infection is the innate immune system, including interferon, natural killer cells (NK) activity and phagocytes (Nash, 1996). Phagocytes are particularly important because their role is to bind to micro-organisms, internalise them and kill them. They respond to primitive recognition signals released from invading microorganisms, although this chemotactic response can be amplified by components of the adaptive immune system (see below). Once an invading microbe has been internalised by a phagocyte, a number of killing mechanisms are used. These include reactive oxygen intermediates (ROIs), reactive nitrogen intermediates (RNIs) and/or antimicrobial peptides (Rook, 1996). Both ROIs and antimicrobial peptides (Rook, 1996; Morimoto *et al.*, 1991; Boman, 1995) have been shown to inactive viruses (Lavelle *et al.* 1973; Michelson & Buckingham 1974) (Fig. 1.3).

Another important part of the innate response is the release and action of interferon. There are two types of interferon involved in the innate response, IFN-α, IFN-β. They are released from virally infected cells and act on other cells by binding to uninfected cells and inducing the production of two enzymes, 2'5'-oligoadenylate synthetase, that activates an endonuclease which cleaves viral mRNA and protein kinase which inhibits protein synthesis (Nash, 1996). Both these mechanisms inhibit the infection of other cells by virus (Fig. 1.3).

NK cells recognise molecules on the surface of virus infected cells, although the exact mechanism is unclear. They can inactivate virus-infected cells by lysis, with or without complement. They also act with complement to opsonise infected cells for phagocytosis (Nash, 1996).

NK cell action can also be enhanced by components from the adaptive immune system (Fig. 1.3).

The alternative complement pathway may also play a role in antiviral immunity. It is an innate mechanism which is activated by the covalent binding of C3b, the key activation component of the complement pathway, to the surface of microorganisms or virus infected cells (Walport, 1996). Some virus infected cells, for example, Epstein-Barr virus infected cells are known to stimulate the alternative complement pathway (Walport, 1996). The induction of the complement cascade leads to opsonisation of target, facilitating phagocytosis and lysis of target cells by the insertion of a hydrophobic plug into the target cell lipid bilayer causing osmotic disruption of the cell (Walport, 1996) (Fig. 1.3).

1.6.2 ADAPTIVE IMMUNITY TO VIRUSES IN VERTEBRATES

The adaptive immune response unfolds and cytotoxic T cells (Tc), helper T cells (Th) and antiviral antibodies appear as infection proceeds (Nash, 1996). Cytotoxic T cells recognise viral antigens associated with MHC I in infected cells and induce cell lysis. T helper cells recognise viral antigens associated with MHC II and release the cytokines IFN-ɣ and TNF (Nash, 1996). IFN-ɣ can activate NK cells and therefore provides an important mechanism for focusing and activating at sites of infection (Nash, 1996). TNF has numerous antiviral activities that are similar to IFN-ɣ but operate through a separate pathway (Nash, 1996). These cytokines also activate phagocytes, stimulating uptake. These processes emphasis the interactions between innate and adaptive immune mechanisms in vertebrate antiviral immunity (Fig. 1.3).

Antiviral antibodies have numerous antiviral properties and can act on both free virus and virus-infected cells. The mechanism of action

on free virus involves blocking of binding and entry into host cells and blocking of viral uncoating mechanisms. Antibody can operate in conjunction with complement via the classical pathway, or on free virus by damaging the viral envelope and blocking viral receptors. Antibody can also act, with or without complement, on infected cells. Antibody and complement can lyse infected cells and opsonise coated virus or infected cells for phagocytosis by phagocytes including macrophages (see above). Antibody/antigen complexes activate the classical complement pathway. Both this pathway and the alternative pathway (outlined above) convert C3 to C3b, the central event of the complement pathway and trigger an enzyme cascade. Complement can opsonise cells (see above), activate leucocytes and lysis target cells. Some viruses, such as murine retroviruses and vesicular stomatitis virus, have been shown to activate the classical complement pathway (Walport, 1996). When antibody alone is bound to infected cells, it enables NK cells, macrophages and neutrophils to kill infected cells by antibody-dependent- cell mediated cytotoxicity (ADCC) (Nash, 1996) (Fig. 1.3).

1.6.3 ANTIVIRAL IMMUNITY IN INVERTEBRATES

Invertebrates do not express clonally derived specific Igs (i.e. antibodies) and therefore are incapable of 'classical' adaptive immunity. Instead, they must rely on innate mechanisms to protect themselves against viral infection. As yet antiviral immunity has not been fully investigated in invertebrates although mechanisms associated with phagocytosis seem likely candidates for antiviral activity, particularly the inactivating properties of antimicrobial peptides and ROIs (Fig. 1.4). An example of the antiviral activity of antimicrobial peptides from invertebrates is that of peptides isolated from the horseshoe crab,

Limulus. Tachyplesin is a cationic peptide, first isolated from haemocyte debris of horse shoe crab, *Tachypleus tridentatus* (Nakamura *et al.*, 1988). The peptide consists of 17 residues and has a unique arginine alpha-amide at the carboxyl terminal end (Nakamura *et al.*, 1988). It inhibits the growth of Gram negative and positive bacteria at low concentrations and can form a complex with bacterial lipopolysaccaride (Nakamura *et al.*, 1988). Tachyplesin I also appears to have antiviral properties (Morimoto *et al.*, 1991). It has the capacity to inhibit multiplication of HIV *in vitro* but only at very high concentrations (Morimoto *et al.*, 1991). At 7.5 μg ml^{-1} it suppressed the development of cytopathic effects by more than 70 % in MT-4 cells infected with HIV (Morimoto *et al.*, 1991). Three isopeptides of tachyplesin I have now been discovered, tachyplesin II and polyphemusin I and II, in the haemocytes of *T. tridentatus* and *Limulus polyphemus*. These peptides have antiviral activity against some enveloped viruses. All inactivate vesticular stomatitis virus (VSV) and tachyplesin I slightly inactivates influenza A (Murakami *et al.*, 1991). However, herpes simplex, adenovirus 1, reovirus 2 and poliovirus are resistant (Murakami *et al.*, 1991). It is not known whether these peptides are capable of inactivating native pathogens (Fig. 1.4).

The production of reactive oxygen species by stimulation of phagocytic cells is a phenomenon that occurs in mammals (Babior *et al.* 1973; Gabig & Babior 1981; Fridovich 1978), fish (Secombes *et al.* 1988; Higson & Jones 1984; Bayne & Levy 1991; Solem *et al.* 1995), molluscs (Dikkeboom *et al.* 1988; Adema *et al.* 1991) and crustaceans (Bell & Smith 1993). The respiratory burst is catalysed by NADPH oxidase (Babior *et al.* 1973). This is a membrane bound enzyme which catalyses the conversion of oxygen to superoxide ions (Babior *et al.* 1973). The oxygen free radicals produced spontaneously dismutase, or are catalysed by the enzyme

superoxide dismutase (SOD), to give hydrogen peroxide (Fridovich 1978). However, although the respiratory burst has been shown to occur in crustaceans (Bell & Smith 1993) it is not known whether it has antiviral properties in these animals (Fig. 1.4).

Another possible mechanism for antiviral immunity is the alternative complement pathway which can be activated by virus or virally infected cells in vertebrates (see above). Recent research has revealed homologues of C3 in the solitary ascidian, *Halocynthia roretzi* (Nonaka *et al.*, 1999) and the purple sea urchin *Stronglyocentrotsus purpuratus* (Alsharif *et al.*, 1998). In, addition, alpha 2-macroglobulin, an important defence protein in invertebrates, has properties analogous to C3 (Enghild *et al.*, 1990). The discovery of these molecules in diverse invertebrate groups suggests the alternative complement pathway exists in at least some invertebrate groups. The role of another enzyme cascade from invertebrates, the prophenoloxidase system, in antiviral immunity is not known. Although the proPO cascade is known to release recognition molecules that opsonise bacteria and facilitate phagocytosis (Smith & Söderhäll, 1983; Söderhäll & Smith 1986; Johansson & Söderhäll, 1989; Söderhäll & Cerenius, 1992) there has been no research to date to determine whether these molecules are capable of opsonising viral pathogens (Fig. 1.4).

The lack of knowledge regarding antiviral mechanisms in crustaceans emphasises the need for new technologies, particularly cell cultures used in conjunction with molecular techniques, to investigate antiviral immunity in crustaceans and aid the search for novel, therapeutic antiviral compounds.

1.7 AIMS AND OBJECTIVES OF THIS STUDY

The main aim of this study was to use a model system to develop techniques to investigate a viral disease in crustaceans to facilitate disease diagnosis and, importantly, to study virus - host interactions at the cellular and population level. The model system selected for use in the present study was the viral pathogen P virus, a reovirus, and its native host, the swimming crab *Liocarcinus depurator*. There are numerous advantages in using a model system, because it is easy to maintain in temperate water aquaria and poses no problems of environmental contamination. In addition, *L. depurator* is very abundant in the North Sea and other european waters and forms part of the by-catch of haddock fisheries, allowing large numbers to be obtained on a regular basis from local fisherman. P virus is also readily purified from experimentally infected individuals and has been fully characterised (Montanie, 1992; Montanie *et al.*, 1993) for a variety of experimental procedures.

The P virus described by Vago (1966) was the first marine invertebrate virus to be discovered. It infects haemocytes, connective tissue and cells of the haemopoietic organ. Virions produce numerous large fuchsinophilic and crystalline arrays 6-10 μm long and 1-3 μm wide (Bonami, 1973; 1980; Bonami *et al.*, 1976). The symptoms of this infection are a gradually increasing level of paralysis and darkening of the exoskeleton. Trembling of the legs occurs after 6 days and continues up to nine days when the crab becomes quiescent. General paralysis follows. The mortality level is 70-85 % with death usually occurring after approximately fifteen days. This virus was discovered in a wild population but also caused the disease when healthy animals were inoculated with a homogenate of infected crab tissue (Bonami, 1980).

P virus is icosohedral in shape, 50-70 nm in diameter with a double protein coat and double stranded RNA genome (Montanie *et al.*, 1993). The buoyant density of full virions 1.29g ml^{-1} (Montanie, 1992) and four major polypeptides have been identified 120, 94, 32, and 24 kD which are thought to be associated with the protein coat (Mari, 1987).

A cDNA library has been constructed for P virus (Montanie, 1992). Briefly, the double stranded RNA genome is denatured then polyadenylated, these positive and negative sense RNA strands are denatured and complementary DNA is synthesised using reverse transcriptase (Montanie, 1992). A double stranded cDNA helix is produced by digesting the RNA with an RNase and using a DNA polymerase to synthesise a new cDNA chain (Montanie, 1992). This cDNA is purified and quantified before being cloned by ligating in a plasmid vector, pUC18 at the Sma 1 site (Montanie, 1992). Several transformations are made and tested on a selective medium containing Ampicillin, X-gal and IPTG (Sambrook *et al.*, 1989). The cDNA library of the P virus genome consists of a bank of 201 clones (Montanie, 1992).

A number of digestions with restriction endonucleases of this cDNA have revealed 8 apparently different inserts (Montanie, 1992). Hybridisation reactions with the viral genome, using inserts as probes labelled with the chemical label digoxigenin, revealed three clones that hybridised with the viral genome (Montanie, 1992). These were the inserts BK0.7c (E2), BK0.7a (B3) and BK0.5 (D6), BK refers to the digestion to remove the insert, at the sites of Bam HI and Kpn I; the number refers to the size of the insert in kilobase pairs and the term in brackets is a reference number used by Montanie (1992). There is a great deal of potential to use these inserts as gene probes to be used as a diagnostic tool

to detect the presence of P virus both *in vivo* and *in vitro* in *L. depurator.*

L. depurator , the host organism, is widely distributed throughout the Mediterranean, east Atlantic and the North Sea (Christiansen, 1969). Several aspects of its biology have been studied including habitat (Haywood 1990), reproduction, moulting (Mori & Zunino, 1987) and feeding ecology (Hall *et al.*, 1990). *L. depurator* inhabits soft, sandy and mixed bottoms from the low water mark to approximately 450 m and grows to a maximum carapace size of around 54 mm (Haywood, 1990). In the Mediterranean, the onset of sexual maturity occurs at a carapace size of 30 mm in males and 24 mm in females (Mori & Zunino, 1987), which is larger than sizes reported from the Atlantic where maturity appears to occur at a size of greater than 20 mm (Hall *et al.*, 1990). Moulting occurs throughout the year, although, moulted females are most frequently observed between April and October (Mori & Zunino, 1987). This species feeds upon a wide range of prey including crustaceans, polychaetes, molluscs and algae (Hall *et al.*, 1990).

To address the lack of suitable techniques to investigate viral diseases in crustaceans, the main objectives of this study are to construct gene probes to P virus and develop a cell culture system for crustacean haemocytes. These techniques will then be used to investigate aspects of P virus infection *in vivo* and *in vitro*. Specifically, the project objectives are:

1. Propagate P virus *in vivo* in *L. depurator* from the North Sea using clarified suspensions of tissue (donated by Dr. Bonami) containing P virus from Mediterranean *L. depurator* .

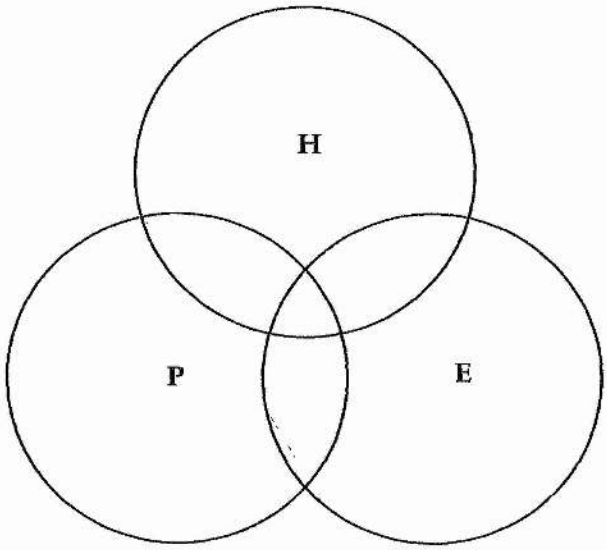
2. Construct gene probes for P virus.

3. Use gene probes to:
   - Test the hypothesis that P virus infects *L. depurator* but not *C. maenas.*
   - Screen animals for presence of P virus.
   - Examine seasonal variation in prevalence of P infection.

4. Develop a cell culture system for decapod crustacean haemocytes.

5. Propagate P virus in *L. depurator in vitro.*

Figure 1.1 Intersecting spheres illustrate the concept of host (H), pathogen, (P) and environment (E) as interactive factors that determine disease. When the size of one sphere is increased (by such factors as increased virulence of a pathogen, perturbations in the environment or nutritional imbalances), incidence or severity of disease increases (indicated by an increased area of overlap of intersecting spheres - middle diagrams). Even in absence of a pathogen, environmental factors can cause disease (lowest diagram) (modified from Snieszko 1973).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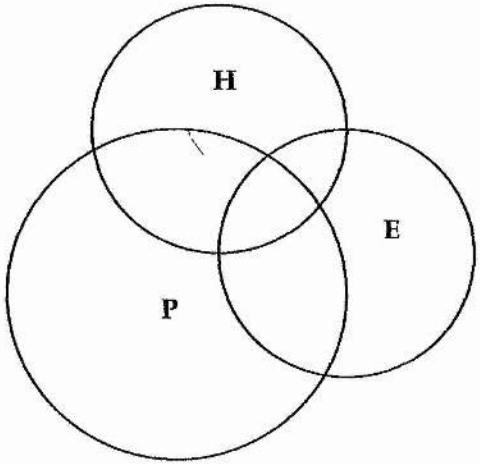
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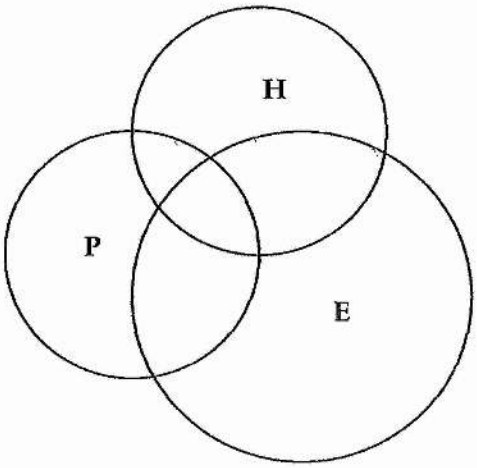
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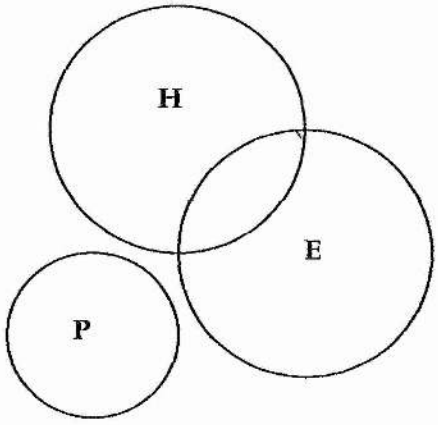
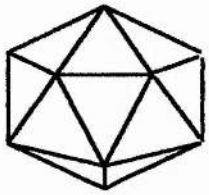

H
E
P

Figure 1.2 Major characteristics of five important virus groups that infect crustaceans.

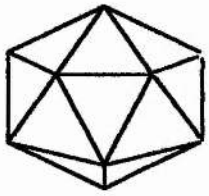
BACULOVIRIDAE

- ds DNA
- enveloped
- nucleus & cytoplasm
- bacilliform
- 40-60 x 200-400 nm



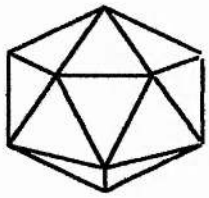
PARVOVIRIDAE

- ss DNA
- non-enveloped
- cytoplasm
- icosahedron
- 18-26 nm



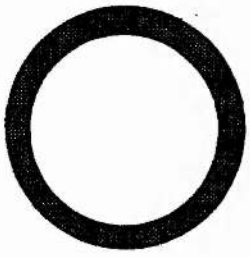
REOVIRIDAE

- ds RNA
- non-enveloped
- cytoplasm
- icosahedron
- 60-70 nm



PICORNAVIRIDAE

- ssRNA
- non-enveloped
- cytoplasm
- icosahedron
- 22-30 nm



BUNYAVIRIDAE

- ss RNA
- enveloped
- cytoplasm
- polymorphic
- 80-200 nm

Table 1.1 Characteristics of viruses from five groups that infect crustaceans. Viruses are listed by abbreviated name, full names (where applicable) are listed in Abbreviations (p. xiii). ? = nucleic acid not determined.

Table 1.1

Virus Family	Virus Name	Virus Size (nm)	Nucleic Acid	Tissue Tropism	Host Family	Host Species	References
Baculoviridae	BP	270 x 50	ds DNA	epithelial cells of hepatopancreas	Penaeidae	*Penaeus duorarum* *P. aztectus* *P. serifus* *P. vannamei* *P. stylirostris* *P. marginatus*	Johnson & Lightner, 1988. Johnson, 1988b.
Baculoviridae	MBV	325 x 75	ds DNA	epithelial cells of mid-gut and hepatopancreas	Penaeidae	*P. monodon* *P. kerathurus* *P. merguiensis* *P. semisulatus*	Johnson & Lightner, 1988. Fukuda *et al.*, 1988.
Baculoviridae	BMN	-	ds DNA			*P. japonicus*	Sano *et al.*, 1984.
Baculoviridae	B1	300 - 320 x 75 - 80	ds DNA	connective tissues, haemocytes	Carcinidae	*Carcinus maenas*	Bazin *et al.*, 1974.
Baculoviridae	T2	300 x 80	ds DNA	epithelial cells of hepatopancreas	Carcinidae	*C. maenas*	Mari & Bonami, 1986.
Baculoviridae	T	300 350 x 70 - 80	ds DNA	epithelial cells of mid-gut and hepatopancreas	Carcinidae	*Carcinus mediterraneus*	Pappalardo & Bonami, 1979.
Baculoviridae	B2	280 - 320 x 70 - 80	ds DNA	haemocytes, connective tissues	Carcinidae	*C. mediterraneus*	Mari & Bonami, 1986.

Table 1.1 cont'd.

Virus Family	Virus Name	Virus Size (nm)	Nucleic Acid	Tissue Tropism	Host Family	Host Species	References
Baculoviridae	Baculo-A	285 x 70	ds DNA	epithelial cells of hepatopancreas	Portunidae	*Callinectes sapidus*	Johnson, 1976; 1978; 1983.
Baculoviridae	Baculo-B	335 x 100	ds DNA	haemocytes, haemopoietic tissue	Portunidae	*C. sapidus*	Johnson, 1983; 1984.
Parvoviridae	HPV	22 - 24	ss DNA	hepatopancreas	Penaeidae	*P. merguiensis* *P. semisulcatus* *P. orientalis* *P. esculentus* *P. monodon* *P. penicillatus*	Lightner & Redman, 1985. Lightner, 1985. Lightner *et al.*, 1985. Paynter *et al.*, 1985.
Parvoviridae	PC84	25	ss DNA	hepatopancreas	Carcinidae	*C. mediterraneus*	Mari & Bonami, 1988a.
Reoviridae	RLV	60	ds RNA	hepatopancreas, connective tissues	Penaeidae	*P. japonicus* *P. monodon* *P. vannamei*	Lightner *et al.*, 1985. Anderson *et al.*, 1987. Krol *et al.*, 1990.
Reoviridae	W	55 - 60	ds RNA	connective tissue and haemocytes	Carcinidae	*C. maenas*	Mari, 1987.
Reoviridae	W2	57 - 62	ds RNA	connective tissue of hepatopancreas and gills, haemocytes	Carcinidae	*C. mediterraenus*	Mari & Bonami, 1988b. Montanie *et al.*, 1993.

Table 1.1 cont'd.

Virus Family	Virus Name	Virus Size (nm)	Nucleic Acid	Tissue Tropism	Host Family	Host Species	References
Reoviridae	RC 84	70 -75	?	epithelial tubules of hepatopancreas	Carcinidae	*C. mediterraenus*	Mari, 1987.
Reoviridae	P	60	ds RNA	connective tissues, haemocytes and hepatopancreas	Portunidae	*Liocarcinus depurator*	Bonami, 1973; 1980. Montanie *et al.*, 1993.
Reoviridae	RLV	55	ds RNA	connective tissues, hepatopancreas	Portunidae	*C. sapidus*	Johnson & Bodammer, 1975.
Bunyaviridae	CHV	135 - 140	?	hepatopancreatic, epithelial cells	Carcinidae	*C. maenas*	Bang, 1971. Hoover & Bang, 1978.
Bunyaviridae	S	100 - 120 x 200	ss RNA	cardiac tissue, connective tissue of hepatopancreas, haemolymph	Portunidae	*L. depurator*	Bonami & Vago, 1971. Bonami *et al.*, 1975.
					Carcinidae	*C. mediterraneus*	Mari, 1987.
Picornaviridae	IHHN	20 - 22	ss RNA	gills, cuticular epidermis, connective tissue, gonads, haematopoietic tissue	Penaeidae	*P. stylirostris* *P. vannamei* *P. monodon* *P. semisulcatus*	Lightner *et al.*, 1983; 1987. Lu *et al.*, 1989. Lightner, 1985. Brock *et al.*, 1983.

Table 1.1 cont'd

Virus Family	Virus Name	Virus Size (nm)	Nucleic Acid	Tissue Tropism	Host Family	Host Species	References
Picornaviridae	CBV	30	ss RNA	mesodermic and ectodermic cells	Portunidae	*C. sapidus*	Johnson, 1978; 1983; 1984.
Picornaviridae	F	31	?	connective tissue, haemocytes	Portunidae	*L. depurator*	Bonami, 1980.
Picornaviridae	N	24	?	connective tissue, haemocytes	Portunidae	*L. depurator*	Bonami, 1980.

Table 1.2 A summary of the current status of diagnostic methods for major crustacean viruses. (modified from Lightner *et al.*, 1992). Viruses are listed by abbreviated name, full names (where applicable) are listed in Abbreviations (p. xiii).

- = no known published application of technique

+ = application published

++ = application published, reasonable diagnostic use

+++ = application published, most sensitive technique available

C = commercially available kits

r&d = research and development stage

r&d* = to be investigated in the present study

Definitions of methods are TEM = transmission electron microscopy; SEM = scanning electron microscopy; ELISA = enzyme-linked immunoabsorbent assay; PAbs = polyclonal antibodies; MAbs = monoclonal antibodies.

1 = Couch, 1991; Lightner & Redman, 1992; Johnson, 1990

2 = Momoyama *et al.*, 1983; Momoyama & Sano, 1989

3 = Lightner & Redman, 1992; Mari *et al.*, 1993; Poulos *et al.*, 1994b

4 = Lightner & Redman, 1992; Mari *et al.*, 1995

5 = Mari & Bonami, 1988a

6 = Bonami *et al.*, 1976; Montanie, 1992; Montanie *et al.*, 1993

7 = Mari, 1987

8 = Adams & Bonami, 1991; Krol *et al.*, 1990; Nash *et al.*, 1996

9 = Bonami, 1980; Mari & Bonami, 1988b; Montanie *et al.*, 1992

10 = Johnson, 1978, 1983, 1984

11 = Bang, 1971; Hoover & Bang, 1978

12 = Lightner & Redman, 1992; Mari *et al.*, 1993; Carr *et al.*, 1996

13 = Bonami & Vago, 1971; Bonami et al., 1975

Table 1.2

	Virus												
Method	BP[1]	BMN[2]	MBV[3]	HPV[4]	PC84[5]	P[6]	RC84[7]	RLV[8]	W[9]	CBV[10]	CHV[11]	IHHN[12]	S[13]
Light Microscopy	++	++	++	++	-	+	-	-	+	+	-	-	-
TEM	+	++	+	+	++	++	++	++	++	++	++	+	++
SEM	-	-	-	-	-	-	-	-	-	-	-	-	-
Histopathology	++	++	++	++	++	+	++	+	+	++	++	++	+
Enhancement	++	-	++	++	-	-	-	-	-	-	-	++	-
Bioassay	+	+	-	-	-	-	-	-	-	-	-	+++	-
Fluorescent Antibody	+	++	-	-	-	-	-	-	-	-	-	r&d	-
ELISA with PAbs	+	-	-	-	-	-	-	-	-	-	-	-	-
ELISA with MAbs	+	-	-	-	-	-	-	-	-	-	-	++	-
Gene Probes	+++	-	+++	+++	-	*r&d	-	-	-	-	-	+++/C	-

Figure 1.3 A summary of the major antiviral immune responses in mammalian vertebrates. Abbreviations used in the diagram are shown in **bold**.

Vertebrates have innate **(green and blue arrows)** and adaptive **(red arrows)** immune responses to virus infection. A detailed explanation is presented in section 1.6.1.

Phagocytes **(PHAG)**, including neutrophils and macrophages, can phagocytose free virus or virus opsonised by the complement (alternative or lectin) pathway **(blue arrows)**. Killing mechanisms used in phagocytosis are reactive nitrogen intermediates **(RNIs)**, reactive oxygen species **(ROSs)** or antimicrobial peptides **(PEP)**. The alternative complement pathway can be stimulated by free virus or infected cells **(green, dotted arrows)** and opsonises viruses or lyses infected cells. If cells become infected they release interferon (IFN-α and β), which protect other cells **(green arrows)**. Natural killer (NK) cells can lyse infected cells by binding to receptors on the cell surface without antibody, although the mechanisms are unclear **(green arrows)**.

Virus is prevented from entering cells by **IgA** (immunoglobulin A) at mucosal surfaces. Other antibodies can bind to free virus forming antigen/antibody complexes that stimulate the classical complement pathway **(red, dotted arrow)**. Antibodies can lyse infected cells directly or via cytotoxic cells **(CT)** by **ADCC** (antibody dependent cell-mediated cytotoxicity). **MHC-1** presents antigenic material at the cell surface to which cytotoxic T lymphocytes **(Tc)** can bind and lyse infected cells. T helper cells **(Th)**, produce interferon γ, which protects other cells; and IL-2 (amongst other cytokines) stimulate cytotoxic T lymphocytes **(Tc)**.

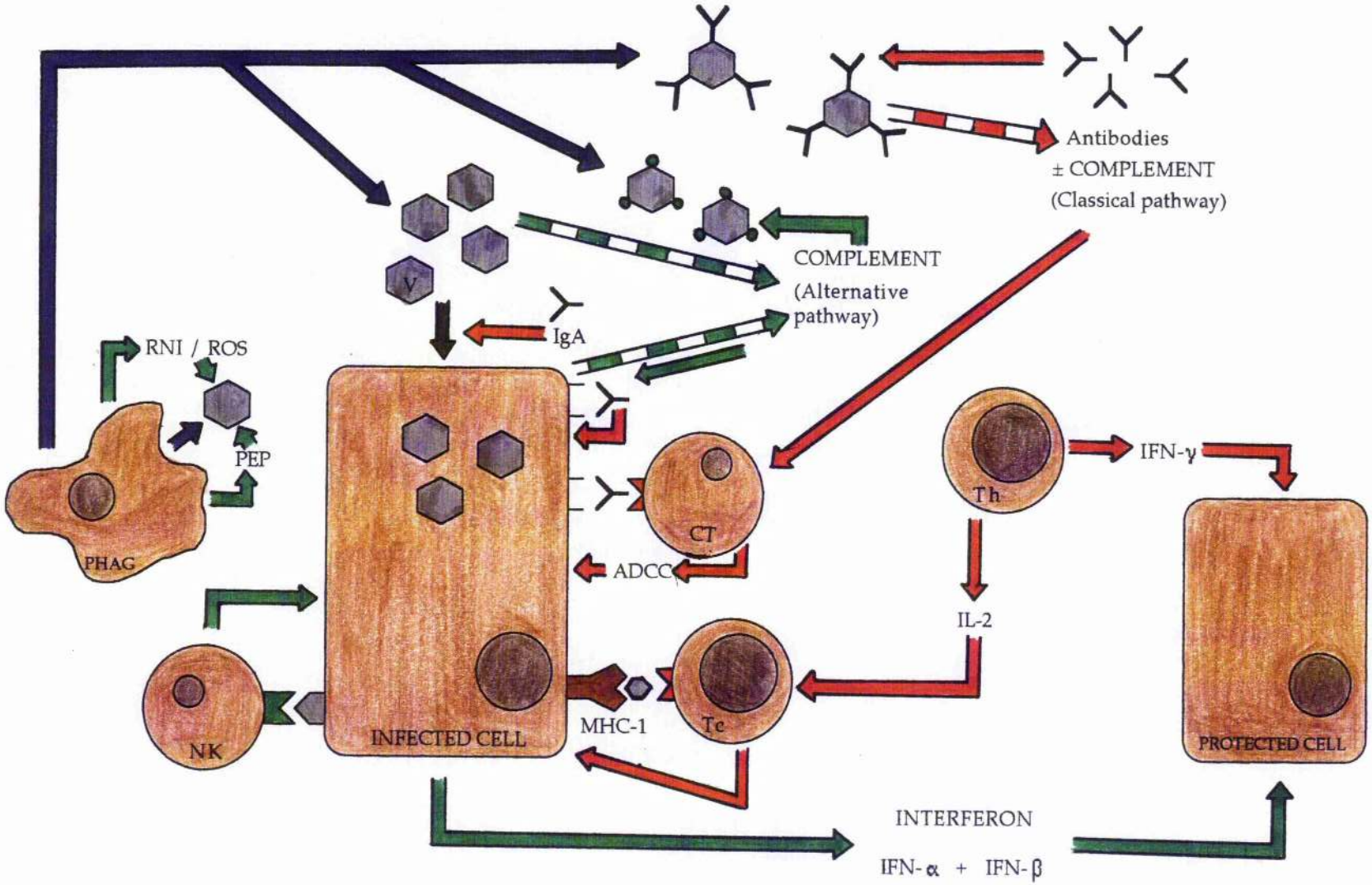
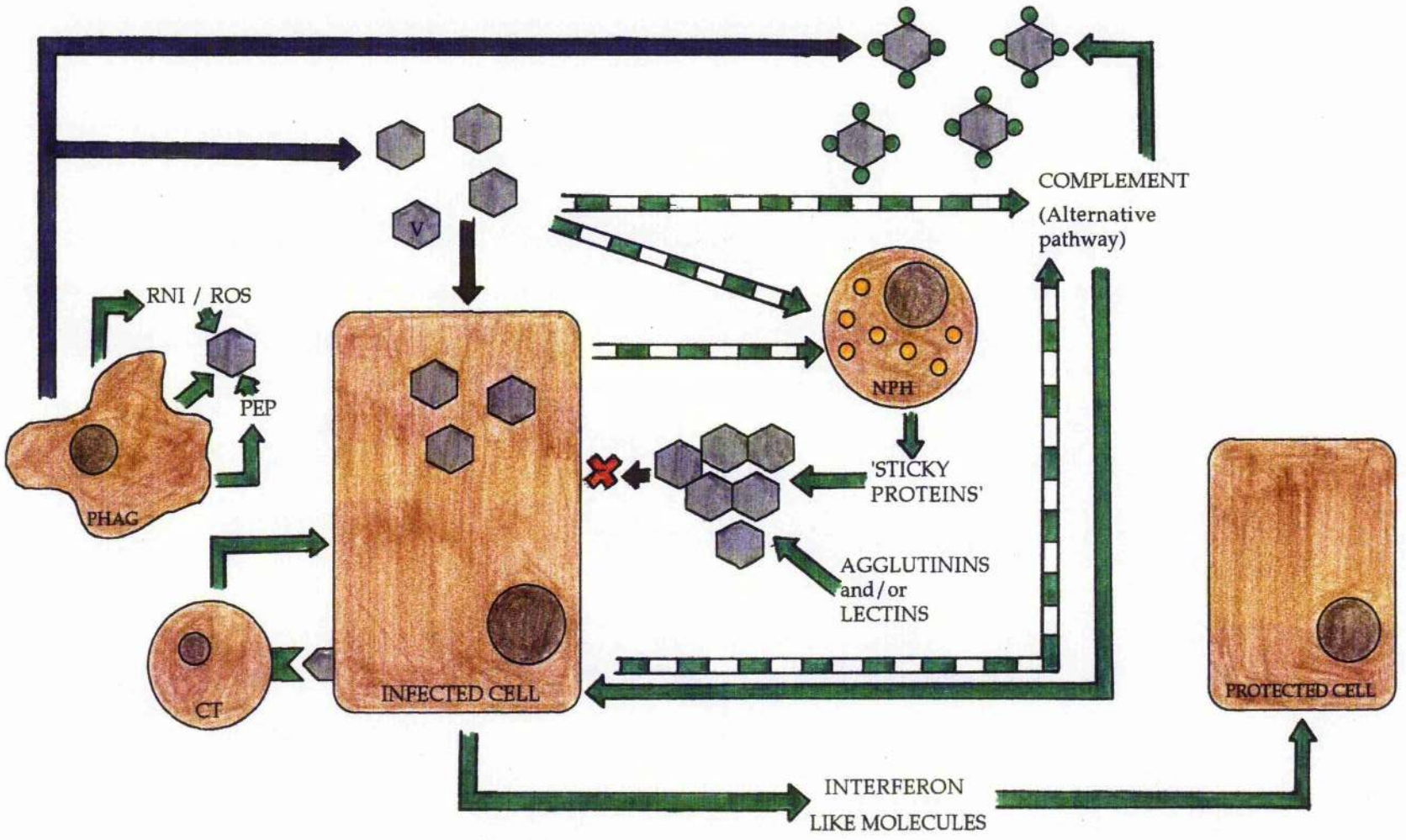

Antibodies
± COMPLEMENT
(Classical pathway)
COMPLEMENT
(Alternative
pathway)
IgA
RNI / ROS
PEP
PHAG
CT
ADCC
Th
IFN-γ
IL-2
NK
INFECTED CELL
MHC-1
Tc
PROTECTED CELL
INTERFERON
IFN-α + IFN-β

Figure 1.4 Theoretical antiviral mechanisms for invertebrates. To date no antiviral immune responses have been fully investigated. Abbreviations used in the diagram are shown in **bold.**

Since invertebrates lack true adaptive i.e. clonally derived lymphocyte-mediated immunity, antiviral immunity is innate **(green arrows)**. Phagocytic haemocytes **(PHAG)**, could phagocytose free virus or opsonised virus **(blue arrows)**. Likely killing mechanisms include reactive nitrogen intermediates **(RNIs)**, reactive oxygen species **(ROSs)** or antimicrobial peptides **(PEP)**. Cytotoxic cells **(CT)** could bind to receptors of infected cells and lyse the cells **(green arrow)**. Infected cells may produce interferon like molecules to protect other cells (green arrow).

Non-phagocytic haemocytes **(NPH)**, could be stimulated by free virus or infected cells (dotted green arrows) to produce **'sticky proteins'** (e.g. proPO proteins or clotting factors). These **'sticky proteins'** could aggregate virions, preventing attachment to host cells (red cross). **Agglutinins and/or lectins,** present in the haemolymph may also produce a similar response.

The alternative complement pathway may exist in invertebrates. It could be activated by free virus or infected cells **(green, dotted arrows)** and may opsonise free virus or lyse infected cells **(green arrows)**.


COMPLEMENT
(Alternative
pathway)
V
RNI / ROS
PEP
PHAG
NPH
'STICKY
PROTEINS'
AGGLUTININS
and/or
LECTINS
INFECTED CELL
CT
PROTECTED CELL
INTERFERON
LIKE MOLECULES

Chapter 2

IN VIVO PROPAGATION OF P VIRUS

2.1 INTRODUCTION

The first marine virus reported was an isolate from the swimming crab, *Liocarcinus depurator* (Portunidae, Decapoda, L. 1758, previously *Macropipus depurator*) from the Mediterranean coast of France (Vago, 1966). This virus was designated P virus and since its discovery, it has been extensively characterised and assigned to the family Reoviridae (Bonami, 1973; 1980; Bonami *et al.*, 1976; Montanie, 1992; Montanie *et al.*, 1993). *L. depurator* , the host organism, is widely distributed throughout the Mediterranean, east Atlantic and the North Sea (Christiansen, 1969). Several aspects of its biology have been studied including habitat (Haywood, 1990), reproduction, moulting (Mori & Zunino, 1987) and feeding ecology (Hall *et al.*, 1990).

In common with other decapods, *L. depurator* has three types of circulating haemocytes, hyaline, semi granular and granular cells (Söderhäll & Smith, 1983). In most decapods, haemocytes have a number of functions including blood coagulation, agglutinin formation, and the storage and distribution of carbohydrates, reviewed by Bauchau (1981). They are also involved in host defence and have phagocytic, encapsulating and lytic activities (Smith & Söderhäll, 1986; Smith & Chisholm, 1992; Söderhäll & Cerenius 1992). Therefore, total haemocyte counts can provide information on the immunocompetence and physiological condition of the individual. Total haemocyte counts are particularly useful to examine the effects of pathogenic challenge (Smith & Ratcliffe, 1980; Cornick & Stewart, 1968; McKay *et al.*, 1969; Persson *et al.*, 1987; Söderhäll *et al.*, 1988; Lorenzon *et al.*, 1999). In the shore crab, *C. maenas*, the injection of bacteria resulted in haemocytopenia (Smith & Ratcliffe 1980), although numbers recover after 24 hours. Similar haemocytopenia has also been observed in the lobster, *Homarus*

americanus (Cornick and Stewart 1968) and the crayfish, *Parachaeraps bicarinatus* (McKay *et al.*, 1969) following injection with bacteria and erthyrocytes respectively. In addition, the crayfish *Pacifastacus leniusculus* exhibited haemocytopenia when infected with the fungus, *Aphanomyces astaci* (Persson *et al.*, 1987; Söderhäll *et al.*, 1988). Similar results have been observed for several marine crustaceans (Lorenzon, 1999).

The histopathology of P virus infection has been extensively studied in *L. depurator* from the Mediterranean (Bonami, 1973; 1980; Bonami *et al.*, 1976) and its biochemical and physical properties have been elucidated (Mari, 1987, Montanie, 1992; Montanie *et al.*, 1993). However, it is not known if the pathogen can be transmitted to other populations of *L. depurator*, from different geographical locations and what effects it has on such populations. Therefore, the aims of this chapter were to (a) to propagate P virus, initially isolated from Mediterranean populations of *L. depurator*, in individuals collected from the North Sea, (b) to determine whether P virus can infect other species of crab by attempting to propagate P virus in a closely related crab, the shore crab *C. maenas* and (c) assess the effect of P virus on the total haemocyte numbers of these two species following infection.

2.2 MATERIALS AND METHODS

2.2.1 Animals

All animals used in experiments were collected in from St. Andrews Bay, North Sea. Swimming crabs, *L. depurator*, were collected in otter trawls and shore crabs, *C. maenas*, were collected in creels (Fig 2.1

& 2.2). The animals were maintained in tanks with a continuous flow of sea water (salinity = 32‰, temperature = 10° C ± 0.64) and aeration. The crabs were fed once per week with chopped herring. Only healthy, male, inter moult animals (carapace width = 34 - 54 mm) were selected for experiments.

2.2.2 Virus purification

To propagate P virus *in vivo*, tissue samples from infected Mediterranean *L. depurator* were prepared by homogenising with a glass piston homogeniser. Hepatopancreas and gills (100 mg) from each crab were homogenised in 400 µl of sterile TN buffer (0.4 M NaCl, 0.02 M Tris-HCL, pH 7.4; Appendix 1) at 4° C and centrifuged at 3 000 g for 10 min. The supernatant, excluding the fat layer on the surface, was transferred to a new tube and stored at -20° C until required.

2.2.3 Transmission electron microscopy

To determine whether hepatopancreas and gill homogenates contained P virus, samples were negatively stained with 2.0 % phosphotungsteic acid. Grids were prepared by placing in a small bottle, rinsing with acetone and sonicating for 1 min. The grids were rinsed twice in distilled water. The chamber in which the grids were coated and the support frame from the chamber were both rinsed with acetone and distilled water before use. After washing, the chamber was filled with distilled water to 1 cm above the support slide. The frame was placed on the support slide and grids carefully placed on the frame, dull side up. To remove dirt from the water surface 1 drop of collodion acetate was placed on the water surface and the resulting film skimmed from the surface. A second drop of collodion acetate was added to the water surface and a

film allowed to develop. The tap at the base of the chamber was opened and the film allowed to settle on the grids. The grids were removed carefully and placed on filter paper in a petri dish and allowed to dry for approximately one hour. To increase the stability of the grids, they were carbon coated in a carbon evaporator. To negatively stain tissue homogenates, a coated grid was inverted on to a drop of homogenate for 3 min, removed and placed onto a drop of 2.0 % phosphotungsteic acid for a further 3 min and allowed to dry before examination under TEM. All grids were observed at a magnification of 12, 000 to detect virions.

2.2.4 Infectivity of P virus from Mediterranean *L. depurator* for North Sea *L. depurator*

The first experiment was conducted using hepatopancreas and gills obtained from Mediterranean *L. depurator* infected with P virus, to determine whether P virus infections could be established in *L. depurator* from the North Sea. A homogenate was prepared from the hepatopancreas and gills using the method described in section 2.2.2. Presence of P virus in this sample was confirmed by TEM on negatively stained samples (described above). This sample was designated P1, and used to inoculate ten fresh *L. depurator* from North Sea in October 1996. Each crab was given an injection containing 0.2 ml of sample P1 via the base of the fifth pereiopod. For controls, ten *L. depurator* were inoculated with 0.2 ml of sterile TN buffer (Appendix 1) by the same technique. These animals were maintained in isolation tanks at ambient aquarium sea water temperature (approximately 13° C) for 40 days. Sea water was changed every 2 days and animals were fed once per week on chopped herring. Hepatopancreas and gills from all animals that died during the experiment were excised, homogenised (see 2.2.2) and examined under

TEM by negative staining (see 2.2.3). Tissues were stored at -20° C for future use.

2.2.5 Effect of P virus on *C. maenas in vivo*

To determine whether P virus could infect a closely related species of crab, specimens of C. *maenas* from the North Sea were inoculated with samples containing P virus from Mediterranean *L. depurator*. The experiment was run in parallel to that described in section 2.2.4. Five *C. maenas* were inoculated with sample P1 in October 1996. Each *C. maenas* was given inoculations of 0.3 ml of P1 via the base of the fifth pereiopod. *C. maenas* were given 0.3 ml injections because this species is larger than *L. depurator*. Five control crabs were given inoculations of 0.3 ml of sterile TN buffer (Appendix 1). Animals were maintained in isolation tanks, at 13° C for 40 days. Water was changed every 2 days and animals fed once a week with chopped herring. Hepatopancreas and gills from all animals were homogenised and checked for presence of P virus by negative staining as described in 2.2.2.

2.2.6 Effect of P virus on total haemocyte numbers

To determine the effects of P virus on total haemocyte counts (THC), *L. depurator* were inoculated with 0.2 ml of sample obtained from the initial propagation described in 2.2.2, named P2. *C. maenas* was inoculated with 0.3 ml of P2 and control animals with 0.3 ml of sterile TN buffer via the base of the fifth pereiopod. Animals were maintained as described in section 2.2.2 at 10 - 15° C. At 7, 14 or 21 days post inoculation, 0.25 ml of haemolymph was extracted from the unsclerotised region of the cheliped directly into 0.25 ml of ice cold anticoagulant (0.45 M NaCl, 0.1 M glucose, 0.03 M tri-sodium citrate, 0.026 M citric acid, 0.01 M EDTA, pH 4.6; Appendix 1). These samples were

diluted further in anticoagulant, 1:5 for *L. depurator* and 1:10 for *C. maenas*. Total haemocyte counts (THC) were determined with an Improved Neubauer haemocytometer and cell counts were expressed as number of cells ml^{-1}.

2.2.7 Haemocyte morphology

Cytospin preparations of haemocytes from all crabs were made by centrifugation for 10 min at 800 g (20°C) on a cytospin 3 (Shandon, Cheshire, UK), using approximately 10^6 cells per slide. The haemocytes were fixed and stained using the Romanovsky method (Diff Quik Staining System, Merz und Dade AG, Switzerland). The slides were dried thoroughly in air then mounted in DePeX (BDH). The cells were photographed using a Lietz Diaplan 20 phase contrast microscope fitted with a Wild Photoautomat MPS45 attachment.

2.2.8 Statistics

Total haemocyte counts are expressed as means ± standard error of the mean for *L. depurator* and *C. maenas* respectively. Student's t-tests on square root transformed data were used to determine significant differences between THC in P and buffer treated animals (Sokal & Rohlf 1981). Accepted level of significance was $P \leq 0.05$.

2.3 RESULTS

2.3.1 Infectivity of P virus from Mediterranean *L. depurator* for North Sea *L. depurator*

Eighty percent of *L. depurator* inoculated with a sample containing P virus purified from Mediterranean crabs died within 40 days post inoculation, although P virus was detected in tissue homogenates of gill and hepatopancreas from only one of these crabs by visualisation under TEM (Fig 2.3). The infected crab died 36 days after inoculation with P virus. Symptoms of infection in *L. depurator* included a loss of appetite, a gradual on set of paralysis, characterised by initial trembling of the legs followed by complete paralysis. By contrast, in control, buffer treated, *L. depurator*, only 40 % of animals died within 40 days of inoculation and mean time of death was 30 days post inoculation. P virus was not observed in any of the *L. depurator* that died within 40 days.

2.3.2 Effect of P virus on *C. maenas*

Thirty percent of *C. maenas*, given injections of P virus, died within 40 days of inoculation. Examination of the tissues from these crabs, however, revealed that no animals had detectable numbers of P virions in their tissues. Two of the ten control *C. maenas* died within 30 days of inoculation but again P virus was not detected in tissues from these animals under TEM.

2.3.3 Effect of North Sea P virus on total haemocyte numbers

When total haemocyte counts (THC) of P treated and control *L. depurator* were compared, no significant difference was observed 7 days post inoculation: THC was $4.11 \pm 1.31 \times 10^6$ ml^{-1} in P treated *L. depurator*

and 5.42 ± 1.38 x 10^6 ml^{-1} in control *L. depurator*. At 14 days post inoculation, THC from P treated *L. depurator* was 3.00 ± 0.85 x 10^6 ml^{-1}, a value significantly lower than THC from control animals, 6.51 ± 0.85 x 10^6 ml^{-1} ($P < 0.05$, Fig. 2.4). THC from P treated *L. depurator* at 14 days post inoculation was 1.6 ± 0.75 x 10^6 ml^{-1}, significantly lower than THC from control *L. depurator*, 6.13 ± 0.51 x 10^6 ml^{-1} ($P < 0.05$, Fig. 2.4). A significant difference was also observed between P treated and control *L. depurator* 28 days post inoculation, THC was 1.6 ± 0.75 x 10^6 ml^{-1} in P treated *L. depurator* and 5.60± 0.98 x 10^6 ml^{-1} in control *L. depurator* ($P < 0.05$, Fig. 2.4).

No significant differences were observed in *C. maenas* inoculated with P or TN buffer only throughout the four week study period, although both treatments resulted in an increase in THC (Fig 2.5). At 7 days post inoculation, THC was 0.71 ± 0.12 x 10^7 ml^{-1} in P treated *C. maenas* and 1.44 ± 0.38 x 10^7 ml^{-1} in control *C. maenas* (Fig 2.5). At 14 days post inoculation, THC was 1.52 ± 0.22 x 10^7 ml^{-1} in P treated *C. maenas* and 1.90 ± 0.27 x 10^7 ml^{-1} in control *C. maenas*. At 21 days post inoculation, THC was 1.52 ± 0.22 x 10^7 ml^{-1} in P treated *C. maenas* and 2.21 ± 0.34 x 10^7 ml^{-1} in control *C. maenas*. At 28 days post inoculation, THC was 1.80 ± 0.14 x 10^7 ml^{-1} in P treated *C. maenas* and 2.32 ± 0.29 x 10^7 ml^{-1} in control *C. maenas* (Fig 2.5).

2.3.4 Cell morphology of haemocytes

L. depurator which suffered haemocytopenia after P virus infection, were found to have haemocytes which were highly vacuolised. This characteristic was rarely observed in control animals, where haemocytes usually appeared intact (Fig. 2.6). Frequently, other infections

were observed in animals with P virus infection. Haemolymph often contained bacterial and protozoan infection (Fig. 2.7).

The haemocytes of healthy *L. depurator*, on the other hand, showed no vacuolisation of haemocytes. Likewise, no vacuolisation was observed in haemocytes from *C. maenas* inoculated with P virus. However, the haemocytes of the P treated *C. maenas* appeared slightly different to those from buffer injected *C. maenas*, with amorphous material visible around the haemocytes (Fig. 2.8).

2.4 DISCUSSION

P virus, from Mediterranean *L. depurator*, was successfully propagated *in vivo* in the swimming crab, *L. depurator* from the North Sea. The presence of P virions in homogenates of gill and hepatopancreas was confirmed in one of the ten *L. depurator*, inoculated with a homogenate containing P virus from Mediterranean crabs, by analysis using transmission electron microscopy. This animal died 36 days after inoculation; findings that contrast with those of Bonami (1973), who experimentally inoculated Mediterranean swimming crabs with P virus. In this case, mortality was 75 - 80 % and P virus could be detected in the tissues of dead animals 15 days post inoculation (Bonami, 1973). Sea water temperatures are lower in the North Sea in October (13° C), when virus was propagated in this study, than in October in the Mediterranean (17 - 20° C). It is possible that difference in temperature between the two studies could be one of the factors that contributed to the variations observed, although other factors, such as the inoculation of crab tissue homogenates from a different geographical location, cannot be discounted.

There was also a large decrease in the number of circulating haemocytes in *L. depurator* infected with North Sea P virus, over a 28 day period. Haemocytopenia has also been observed in *C. maenas* following injections of bacteria, LPS or glucans (Smith & Ratcliffe 1980; Smith & Söderhäll 1983). Similar changes in THC have been noted for the lobster *Homarus americanus* (Cornick & Stewart 1968), the freshwater crayfish, *Parachaeraps bicarinatus* (McKay *et al.*, 1969), *A. astectus* and *P. leniusculus* (Smith & Söderhäll 1983) and in numerous other crustaceans including *Nephrops norvegicus, Mundia rugosa, Paguristes oculatus, Pilumnus hirtellus, Macropipus vernalis, Parthenope massena* and *Ilia nucleus* (Lorenzon, *et al.*, 1999). In *C. maenas*, haemocytopenia occurs in parallel to a rapid clearance of the pathogen from the circulation (Smith & Ratcliffe, 1980), although haemocyte numbers generally recover to pre injection levels after 24h, indicating mobilisation and/or haemopoeisis had taken place (Smith & Ratcliffe, 1980). In the present study, *L. depurator* haemocyte numbers did not recover to restive levels, even after 28 days. Although haemopoiesis is poorly understood in crustaceans, it is possible that infection with P virus may hamper haemopoeisis, leading to the observed reduction in THC.

Interestingly, bacteria and protozoans were sometimes observed in animals infected with P virus. This may be because a reduction in the circulating haemocytes leads to a reduction in the immunocompetence of the host. Haemocytes play an important role in host defence (Bauchau, 1981; Smith & Söderhäll, 1983; Smith & Chisholm, 1992), although *in vivo*, it is not possible to determine the relationship between P virus and other infections because of the many intrinsic and extrinsic factors which could affect the animal.

The haemocytes from P infected *L. depurator*, show a cytopathic effect. In mammals, reoviruses are known to induce apoptosis as part of their cytopathic effect in cultured L929 fibroblasts (Tyler *et al.*, 1995). This results in cytoplasmic vacuolisation very similar to that observed in haemocytes from *L. depurator* infected with P virus in this study. It is not clear whether the vacuolisation observed in *L. depurator* in the present study was directly caused by P virus, which is known to form lesions in cells and tissues due to formation of crystalline arrays (Bonami *et al.*, 1976), or indirectly as a consequence of another opportunistic infection. P virus is usually found in association with S virus, a bunyavirus discovered by Bonami & Vago (1971). The two viruses have been reported to develop in parallel following experimental infection *in vivo* (Bonami, 1973). The inability to determine the cause of pathological symptoms *in vivo* further highlights the need for *in vitro* systems to investigate viral infections.

In contrast to *L. depurator*, haemocytopenia was not observed in *C. maenas* during the present study and P virus could not be detected in tissues of *C. maenas*, indicating failure of P virus to establish infection in this species. Nevertheless, there were some mortalities which could be a result of the inoculation of a homogenate from *L. depurator* which would almost invariably recognised as non-self by *C. maenas* individuals. Indeed, flocculant material was visible in the haemolymph from *C. maenas* which may be remnants of cells utilised in the immune response. Similar flocculant has been observed in *C. maenas* inoculated with bacteria (Smith, 1978). Interestingly, Bonami (1980) found that 11 out of 17 *C. maenas* from the Mediterranean, inoculated with P virus, died with symptoms and histopathological characteristics of P virus infection. Since the study by Bonami (1980), genetic differences have been

discovered between *Carcinus* populations from the Mediterranean and Atlantic and they are now classified as sub-species (Bulnheim & Bahns, 1996). There are also some differences in environmental conditions, such as temperature. These factors may explain variation in pathogenicity between the two studies.

The experiments reported in this chapter demonstrate that P virus from the Mediterranean can be experimentally transmitted to *L. depurator* from the North Sea. However, the presence of virus can only be verified by EM, a technique which is laborious and relies upon subjective identification of viral particles from other structures and residues. Clearly, more rapid and accurate diagnostic methods, which could be used in conjunction with *in vitro* systems, are needed.

Figure 2.1 The swimming crab, *Liocarcinus depurator*. Animal is shown at actual size of 55 mm (carapace width). This species inhabits soft, sandy and mixed bottoms from low water to 450 m. It has a wide distribution, in the Atlantic, Mediterranean and North Sea. Adults range in size from 30 - 55 mm (carapace width).

Figure 2.2 The shore crab, *Carcinus maenas*. Animal is shown at actual size of 70 mm. This species inhabits sandy and rocky shores in the inter-tidal zone and shallow water. It is widely distributed in the Atlantic, Mediterranean, English Channel, North Sea and Baltic. Adults range in size from 40 - 75 mm (carapace width).

Figure 2.3 Transmission electron micrograph showing P virions (arrows) in a homogenate of hepatopancreas and gills from an infected swimming crab, *L. depurator*. Homogenates are prepared using a glass piston homogeniser and centrifuged to remove cell and tissue debris. The purified suspension is negatively stained using phosphotungstenic acid for electron microscopy. Scale bar = 700 nm.

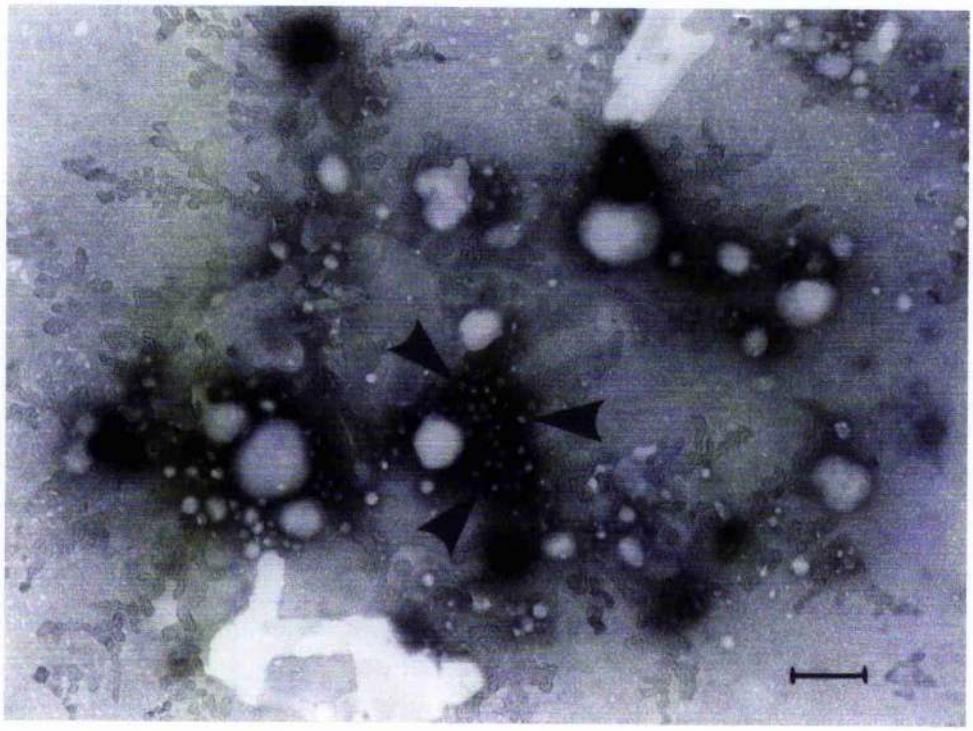


Figure 2.4 Total haemocyte counts (THC) from *L. depurator* inoculated with purified P virus (▨) or sterile TN buffer (■). A significant difference in THC is observed between experimental *L. depurator* (P-inoculated) and control *L. depurator* (buffer-inoculated) at 14, 21 and 28 days post inoculation ($P < 0.05$ = * on figure). No signifcant difference is observed between experimental and control *L. depurator* at 7 days post inoculation (NS on figure). n = 10.

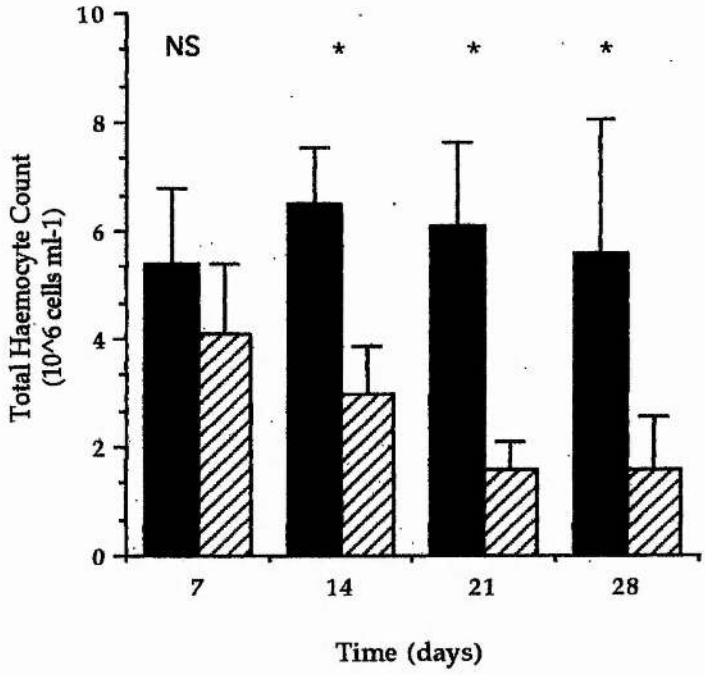

10
8
6
4
2
0
NS
*
*
*
Total Haemocyte Count
(10^6 cells ml-1)
7
14
21
28
Time (days)

Figure 2.5 Total haemocyte counts (THC) from C. *maenas* inoculated with P virus (▨) or TN buffer (■). No significant differences are observed between the two treatments (NS on figure). THC increases over the 28 day period in crabs from both treatment groups. n = 6.

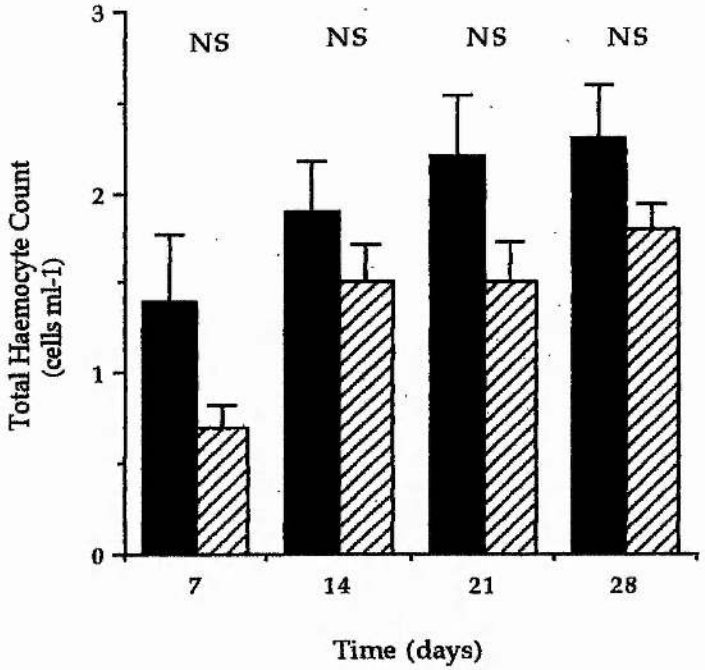

NS
NS
NS
NS
3
2
1
0
Total Haemocyte Count
(cells ml-1)
7
14
21
28
Time (days)

Figure 2.6 Cytospin of *L. depurator* haemocytes 7 days (A and B) and 14 days (C and D) post inoculation.

A. Appearance of haemocytes from animals inoculated with TN buffer 7 days post inoculation. All haemocytes are intact and show no signs of necrosis. Scale bar = 5 μm.

B. Appearance of haemocytes from animals inoculated with P virus 7 days post inoculation. Haemocytes are vacuolised, often around the perinuclear membrane, indicative of reovirus infections (arrows). Scale bar = 5 μm.

C. Appearance of haemocytes from animals inoculated with TN buffer 14 days post inoculation. Again, haemocytes are intact and show no signs of necrosis. Scale bar = 5 μm.

D. Appearance of haemocytes from animals inoculated with P virus 14 days post inoculation. Haemocytes exhibit the same characterised observed after 7 days (arrows) (see B) Scale bar = 5 μm.

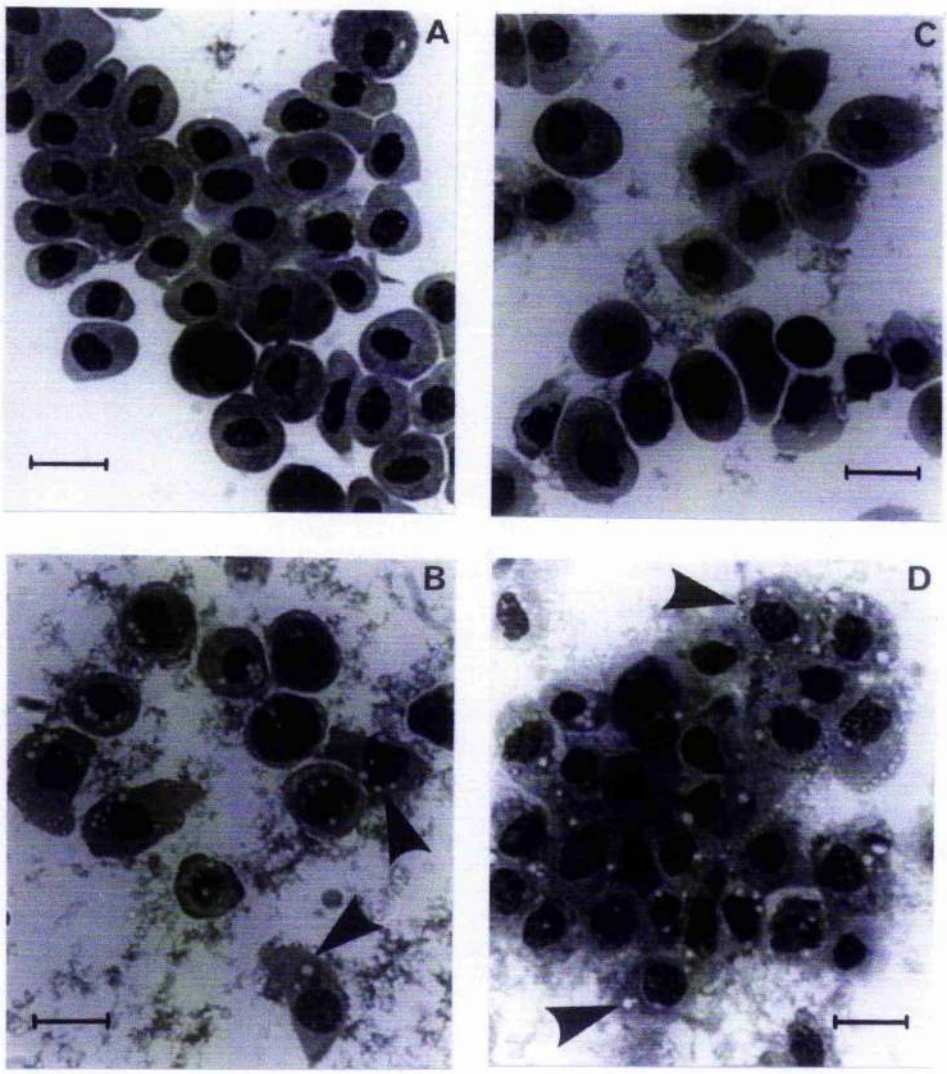

A
C
B
D

Figure 2.7 Cytospin of *L. depurator* haemolymph 21 days post inoculation with P virus. A number of protozoan parasites are observed in the haemolymph, one of which appears to be attempting to phagocytose a haemocyte (arrow A). This amoeba is similar in appearance to *Paramoeba perniciosa*, an amoeba which is responsible for grey crab disease in *C. sapidus* (Johnson 1988a). The animal is also heavily infected with bacteria, which some haemocytes have phagocytosed (arrow B). Scale bar = 5 μm.

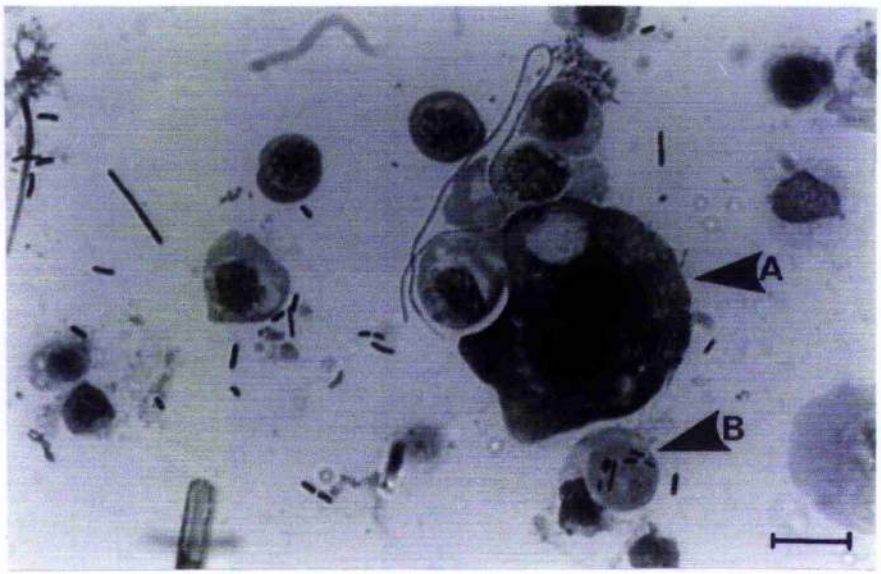
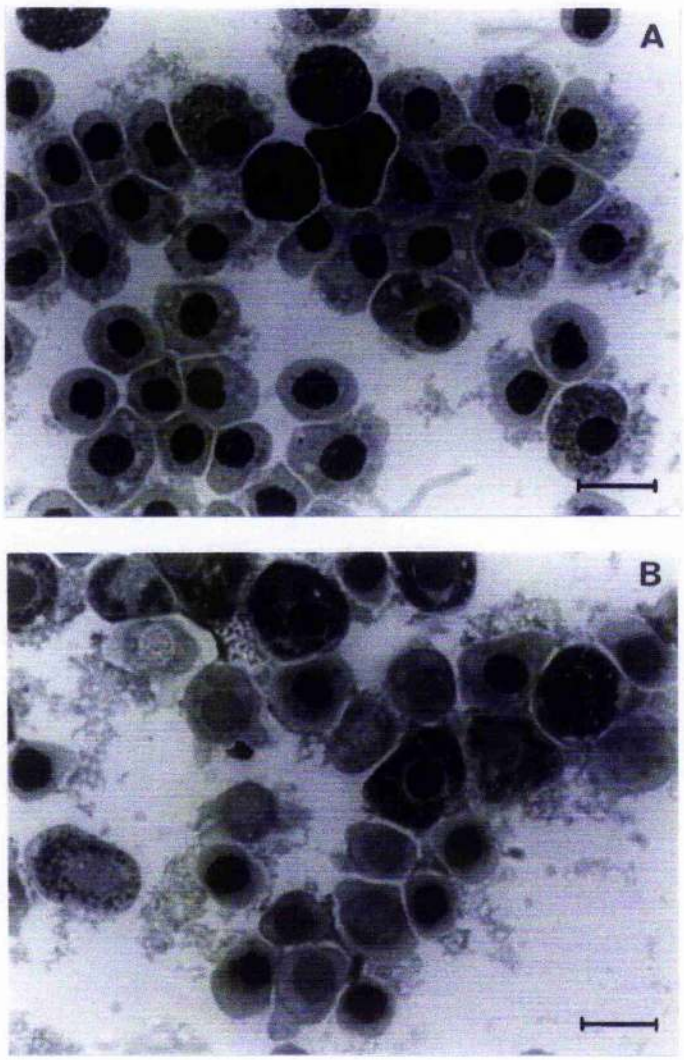

A
B

Figure 2.8 Cytospin of *C. maenas* inoculated with TN buffer (A) or P virus (B) at 7 days post inoculation.

A. Haemocytes appear intact with no signs of necrosis or vacuolisation. Scale bar = 5 μm.

B. Haemocytes appear intact with no signs of necrosis although some flocculant material is visible in the haemolymph. Scale bar = 5 μm.


A
B

Chapter 3

CONSTRUCTION OF A GENE PROBE FOR DETECTION OF P VIRUS IN *L. DEPURATOR*

3.1 INTRODUCTION

In recent years, the development of gene probes has offered considerable promise as diagnostic tools for diseases of viral aetiology and such probes are now widely accepted as amongst the most sensitive and accurate ways of detecting specific viruses in animal tissues. To date relatively few gene probes have been constructed and tested for marine invertebrates. Those for crustaceans have been only prepared for shrimp viruses (Lightner, 1996; Durand *et al.*, 1997; Mari *et al.*, 1998; Durand *et al.*, 1998) where they have found great application in commercial shrimp farming. As yet, none are available for viruses infecting crabs, lobsters or crayfish, even though these animals comprise a major part of the shell fishery in Europe, North America and Scandinavia. Importantly, gene probes need to be developed for those crustacean species which represent useful laboratory models for analyses of the virus-host interaction at the cellular level, to permit evaluation of any innate host defence reactions mounted by crustaceans to viral agents and to facilitate screening of potentially useful anti viral compounds for therapeutic use in aquaculture.

The aim of this chapter was to develop a gene probe for P virus, a reovirus infection of the swimming crab *L. depurator*. P virus has a genome consisting of 12 linear pieces of dsRNA (1 large, 5 medium and 6 small sized segments (Montanie, 1992; Montanie *et al.*, 1993) that has been extensively cloned (Montanie, 1992). Successful cloning of the P virus genome will enable cDNA probes to be constructed to detect P virus in the tissues of infected animals.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Animals were collected and maintained as described in 2.2.2.

3.2.2 Plasmid purification

The genome of P virus was cloned by Montanie (1992). Three of these clones, designated B3, D6 and E2a, were donated by Dr. Bonami for development as gene probes in this study. The clones were supplied in pUC18 plasmid vector contained in *E. coli* (strain DH5-α) on LB Agar plates. A single colony from each plate (containing either B3, D6 and E2) was used to inoculate 5 ml of sterile LB (Luria Bertanic) broth (Fluka, Dorset, UK) and incubated overnight at 37° C in a shaking incubator. A mini preparation kit (Wizard® kit, Promega, Southampton, UK) was used to purify plasmids from bacterial cultures. The overnight culture (5 ml) was pelleted by centrifugation for 5 min at 10 000 g. The supernatant was removed and the tube turned upside down on a paper towel to remove excess liquid. Then, 250 µl of cell resuspension solution (Wizard®) (Promega) was added to the tube and the pellet was resuspended by vortexing. 250 µl of cell lysis solution (Wizard®) (Promega) was added to each tube and mixed by inverting 4 times. The cell suspension was incubated for 1 - 5 min until the suspension cleared. When the solution cleared, 10 µl of alkaline protease solution (Wizard®) (Promega) was added to the cleared lysate and mixed by inverting 4 times before incubating for 5 min. Then, 350 µl of neutralisation solution was added to each tube and mixed by inverting 4 times. The lysate was centrifuged at 12 000 g in a micro centrifuge for 10 min at room temperature.

The cleared lysate was transferred to a spin column (Wizard®) (Promega), after centrifugation, by decanting. Care was taken not to transfer any white precipitate to the spin column. The column was centrifuged at 12 000 g for 1 min at room temperature and the flow through discarded. Then, 750 μl of column wash solution (Wizard®) (Promega) was added to the spin column and centrifuged at 12 000 g for 1 min at room temperature. The flow through was discarded and column re inserted. To wash the column a second time, 250 μl of column wash solution was added and centrifuged at 14 000 g for 2 min at room temperature. The spin column was transferred to a new tube and plasmid DNA eluted by adding 100 μl of nuclease-free water and centrifuging at 12 000 g for 1 min at room temperature.

3.2.3 Extraction of inserts by digestion with restriction endonucleases

The inserts B3, D6 and E2a were extracted from purified plasmid using restriction endonucleases. The fragment D6, corresponding to the large piece of the genome, was extracted with Eco RI (Boehringer Mannheim, Lowes, Sussex, UK), while E2a, corresponding to one of the five pieces of the medium sized fragments of genome, was extracted with Kpn I (Boehringer Mannheim) and Bam HI (Boehringer Mannheim) and B3, corresponding to one of the six small sized fragments of the genome, was extracted with Kpn I. All digests were performed in a final volume of 20 μl using substrate DNA of concentration 2μg ml^{-1} (in sterile deionised water), a ten fold excess of restriction enzyme, a ten fold dilution of incubation buffer (supplied with restriction enzymes by Boehringer Mannheim, see below) and BSA (stock 10 μg ml^{-1} in sterile deionised water) where appropriate.

The insert B3 was extracted from pUC18 plasmid using the restriction enzyme Bam HI. In a sterile tube the following reagents were assembled in order (values in brackets are stock concentrations):

Sterile deionised water	9.0 μl
Buffer L (10 mM Tris-HCL, 10 mM $MgCl_2$	2.0 μl
1 mM dithioerythrythol, pH 7.5)	
BSA (10 $\mu g\ \mu l^{-1}$)	2.0 μl
DNA (2 $\mu g\ \mu l^{-1}$)	5.0 μl
Kpn I (2 000 units)	2.0 μl

These reagents were mixed and incubated at 37° C for 2 hours.

The insert D6 was extracted using the restriction enzyme Eco RI. Reagents were added to a sterile tube in the following order:

Sterile deionised water	11.0 μl
Buffer H, (50 mM Tris-HCl, 100 mM NaCl,	2.0 μl
10 mM $MgCl_2$, 1 mM dithioerythritol; pH 7.5)	
DNA (2 $\mu g\ \mu l^{-1}$)	5.0 μl
Eco RI (5 000 units)	2.0 μl

These reagents were mixed and incubated at 37° C for 2 hours.

The insert E2a was extracted using the restriction enzymes Kpn I and Bam HI. In a sterile tube the following reagents were assembled in order:

Sterile deionised water	1.0 μl
Buffer L	1.0 μl
BSA (10 $\mu g\ \mu l^{-1}$)	1.0 μl
DNA (2 $\mu g\ \mu l^{-1}$)	5.0 μl
Kpn I (2 000 units)	2.0 μl

The above reagents were mixed and incubated at 37° C for 2 hours. The following reagents were added to the tube to complete the double digestion and incubated for a further 2 hours at 37° C:

Sterile deionised water	6.0 µl
Buffer B	2.0 µl
(10 mM Tris-HCl, 10 mM NaCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol; pH 8.0)	
Bam HI (1 000 units)	2.0 µl

The products of digestion were run on a 1 % agarose gel containing ethidium bromide (final concentration 0.5 µg μl^{-1}; Appendix 2) for 40 min at 80 Volts and visualised under UV. The inserts were cut from the gel using a sterile scalpel and placed in a sterile tube for DNA extraction using a DNA recovery kit (Hybaid, Ashford, Middlesex, UK). Binding buffer (400 µl) (Hybaid) was placed in a spin column and a gel slice added (not more than 300 mg per filter). The column containing binding buffer and gel slice was incubated at 55° C for 5 min or until the gel melted. The tube was flicked to mix. The column was centrifuged for 15 - 30 sec to transfer liquid to the catch tube below. The liquid from the tube was discarded and the column re-assembled. Then, 500 µl of wash solution (Hybaid) was added to the spin filter and centrifuged for a further 30 sec, until the filter was emptied of wash solution. This washing step was repeated and the catch tube emptied. The tube was centrifuged for a further 1 min to dry the filter. The spin filter was transferred to a new tube and 25 µl of nuclease-free water added and the DNA resuspended by flicking the tube and centrifuging for 30 seconds to elute the DNA. This step was repeated to increase yield of DNA.

3.2.4 Probe labelling

Each insert, purified as above was labelled with digoxigenin, using the random prime method (Boehringer Mannheim), for use as gene probes. On ice, 15 µl of DNA (0.5 - 3.0 µg), 2 µl of hexanucleotide mix (Boehringer), 2 µl of dNTP mix (Boehringer Mannheim)and 1 µl of Klenow enzyme (Boehringer Mannheim) were placed in a microfuge tube. The reagents were mixed, centrifuged briefly at 12 000 g at room temperature and incubated overnight at 37° C. To stop the reaction, 2 µl of 0.2 M EDTA, pH 8.0 was added to the tube. The labelled DNA was precipitated by adding 2.5 µl of 4 M LiCl and 75 µl of pre-chilled ethanol, mixing well and incubating for 1 hour at -70° C. The tube was centrifuged at 12 000 g for 15 min at room temperature. The DNA pellet was dried briefly under vacuum and dissolved in 50 µl of TE buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.0; Appendix 3).

To quantify the concentration of labelled probe, control labelled DNA (Boehringer Mannheim) was diluted 1:5 to a concentration of 1 µg ml-1. Both control and experimental probes were serially diluted tenfold to 10^{-5}, five times and 1 µl of each of dilution spotted onto positively charged nylon membranes (Boehringer Mannheim) and DNA fixed under UV for 2 min. The membrane was placed in a bag containing 10 ml of 0.5 % blocking solution (0.05 g of blocking reagent (Boehringer Mannheim) in 10 ml of buffer 1; Appendix 3) and incubated for 30 min at room temperature. The membrane was washed in 20 ml of buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5; Appendix 3) for 5 min at room temperature. Antibody conjugate solution was prepared during the washing step by adding 0.5 µl of antibody conjugate solution (Boehringer Mannheim) to 2.5 ml of buffer 1 (Appendix 3) and mixing well. The membrane was incubated in antibody solution for 30 min at room

temperature before washing twice in buffer 1 (Appendix 3) for 15 min at room temperature. Detection solution was prepared by adding 45 μl of NBT (Nitro-Blue Tetrazolium) and 35 μl of X-phosphate to 10 ml of buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5; Appendix 3). The membrane was placed in a bag with 10 ml of detection solution and incubated in the dark until the desired spots appeared. To terminate the reaction the membrane was washed in sterile deionised water. The intensity of control and experimental dilutions were compared to estimate the concentration of DIG-labelled probe. An outline of the procedures involved in probe construction is presented in Fig. 3.1.

3.2.5 Dot blot hybridisation

Probes were tested using tissue homogenates from *L. depurator* known to be infected with P virus (obtained from *in vivo* propagation of virus described in 2.2.2), healthy *L. depurator* or *C. maenas* (prepared as described in section 2.2.2), purified samples of P virus prepared as described in Montanie *et al.*, (1993) or purified samples of W2, a closely related virus that infects *C. maenas* (Montanie *et al.*, 1993) (donated by Dr. Bonami). For dot blot analyses, the homogenates and purified samples were thawed on ice and incubated with proteinase K (0.5 μg ml^{-1} in sterile deionised water, final concentration; Appendix 3) for 1 h at 37° C followed by incubation with N-lauryl sarcosine (0.5 % in sterile deionised water, final concentration; Appendix 3) for 1 h at 67° C. The samples were diluted 1:2 in denaturing buffer (50 % formamide; 6 % formaldehyde; 1X MOPS = 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0; final concentrations; Appendix 3), denatured for 5 min at 67° C and chilled immediately on ice. A positive control was prepared

using plasmid containing the appropriate insert (i.e.. B3, D6 or E2a), diluted tenfold in sterile deionised water, denatured for 10 min at 100° C and then chilled immediately to 4° C. All samples were centrifuged briefly and 1 μl aliquots dotted onto a positively charged membranes (Boehringer Mannheim). The membranes were dried for 1 - 1.5 h at 50° C and fixed under UV light for 4 min. Pre-warmed high SDS hybridisation buffer (50 % formamide; 2X SSC = 0.3 M NaCl, 0.03 M Sodium citrate, pH 7.0; 0.1 M sodium phosphate; 2 % blocking solution; 0.5 % N-lauryl sarcosine; 7 % SDS; Appendix 3) was added to each membrane (25 ml per 100 cm^2) in a bag and all membranes were incubated in a rocking incubator (Hybaid) at 42° C for 2-4 h. Next, the pre-hybridisation solution was replaced with hybridisation buffer (30 ng ml^{-1} of denatured probe, either B3, D6 or E2a, in high SDS hybridisation buffer) and the membranes incubated overnight at 42° C in a rocking hybridisation oven (Hybaid). The following day, the membranes were washed twice in 2X wash (2X SSC; 0.1 % SDS, pH 7.0; Appendix 3) for 15 min at room temperature with shaking, followed by two washes in 0.5X wash (0.5X SSC, 0.1 % SDS, pH 7.0; Appendix 3) for 15 min at 68° C. They were incubated in 1 % blocking solution (10 % blocking solution in maleic acid buffer; Appendix 3) for 1 h at room temperature with shaking and then incubated in alkaline phosphatase-labelled sheep anti-digoxigenin antibody conjugate without shaking (anti-DIG-AP, Boehringer Mannheim) (1:5000 in 1 % blocking solution) for 1h at RT. This was followed by two washes in washing buffer (0.1 M maleic acid; 0.15 M NaCl; 0.3 % Tween 20; pH 7.5; Appendix 3) with shaking at room temperature for 15 min before equilibrating in detection buffer (45 μl of NBT and 35 μl of X-phosphate to 10 ml of buffer 3; Appendix 3), for 5

min at room temperature. The membrane was incubated in the dark, at room temperature, until the desired spots appeared.

3.2.6 Production of probe by PCR

Having established that probe E2a gave the strongest signals and lowest background, it was selected for further development. The insert E2 was sequenced by the dye-dideoxy terminator method using M13 forward and reverse universal primers (Societe ESGS, Genopole, France). Following sequence analysis, two primers, R1 and F1 were designed to the E2 sequence. The sequence of F1 is 5'CAG ATG CTG ATG TTT CAG AG3' and the sequence of R1 is 5'CCA GCA AGT GTC TAA ACA TA3'. Amplifications were performed using "Ready-to-go" beads (Promega). To each tube, containing a PCR bead, 0.5μl of each primer, 0.5μl DNA (100 ng) and 23.5μl of water were added at room temperature (1.5 units of *Taq* DNA polymerase in 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$ and 200μM of each dNTPS, BSA). The tube was vortexed and centrifuged briefly. The thermal cycling conditions were 30 cycles of 1 min 30' at 94.0°C, 1 min 30' at 55.0°C and 2 min at 72.0°C followed by a final cycle of 7m at 72.0°C (Hybaid thermal cycler). The products were checked by running the complete sample on a 1.5 % agarose gel (see 3.2.3) and amplified product extracted using a DNA extraction kit (Hybaid). The PCR product was labelled for use a gene probe as described in 3.2.4 and named E2b. The E2b probe was then tested by dot blotting as described in section 3.2.5.

3.2.7 *In situ* hybridisation

In situ hybridisation was used to further test the suitability of E2b probe. Pieces of tissue (ca 0.5 cm^3) from specimens of *L. depurator,*

known to be infected with P virus (see 2.2.1) were fixed for 24 h in modified Davisons fixative (Hasson *et al.*, 1997, Appendix 1), dehydrated through a series of alcohols, cleared in xylene and finally embedded in paraffin wax. Sections of ca 0.6 μm were cut and mounted on polysine, positively charged, slides (BDH). Sections were dewaxed by rinsing twice in xylene (5 min), rehydrated through a series of alcohols to distilled water and then incubated for 15 min at 37° C with 250 μl of proteinase K per slide (0.5 μg ml^{-1} see 3.2.5 and Appendix 3) in an Omnislide thermal cycler (Hybaid). The slides were rinsed twice in RNase-free water for 10 min at room temperature and then incubated for 1 h at 42° C with 250 μl of hybridisation buffer per slide (Appendix 3). The pre-hybridisation buffer was replaced with 100 μl of hybridisation buffer containing E2b probe (30 ng ml^{-1} final concentration) and incubated initially for 10 min at 68° C and then 16h at 42° C.

After hybridisation, the slides were washed in 2X wash (2 x 20 min, see 3.2.5 and Appendix 3) and prewarmed (42° C) 0.1X SSC (Appendix 3), followed by a wash in 2 mM $MgCl_2$, 0.1% Triton 100X (1 x 30 min); then blocked with 1% blocking solution (10 % blocking solution diluted in maleic acid buffer; Appendix 3) for 30 min at 37° C in a humid chamber. Anti-DIG-AP (Boehringer Mannheim) was diluted 1:1000 in 1 % blocking solution and incubated for 30 min at 37° C in a humid chamber. Slides were then washed with buffer 1 (see 3.2.5 and Appendix 3), 2 x 5 min at room temperature and equilibrated in buffer 3 (Appendix 3) for 5 min at room temperature. Development solution was prepared immediately before use (see 3.2.5 and Appendix 3). Slides were incubated overnight with 250 μl per slide of the development solution in a dark, humid chamber at room temperature. After incubation (maximum of 16 h), the slides were washed with distilled water, counter stained with 0.1 %

aqueous eosin and mounted in a glycerol mounting media (Sigma). Negative controls were prepared in parallel by incubating sections without probe or antibody. Each slide was examined and photographed under bright field optics of a Leitz Diaplan photo microscope with Wild Photoautomat attachment). An outline of the major steps in *in situ* hybridisation is presented in Fig. 3.2.

3.3 RESULTS

3.3.1 Production of probes by digestion with restriction endonucleases

Three cDNA inserts, designated D6, E2a and B3 were successfully extracted from pUC18 vector. The restriction enzyme Eco RI was used to extract D6; Kpn I was used to extract B3 and a double digestion using Kpn I and Bam HI was used to extract E2a (Fig. 3.3)

3.3.2 Detection of virus by dot blots using B3, D6 and E2a probes

All probes produced reactions with positive controls, purified plasmid DNA containing D6, E2a or B3. The probes E2a and D6 detected P virus in a purified sample (lane B) and a homogenate of infected tissue that was known to contain P virus by negative staining with TEM (Fig 3.4i and 3.4ii; lane D). However, there were also non-specific reactions with homogenates of uninfected *L. depurator* tissue (lane F) and tissue from *C. maenas* (Fig 3.4i and 3.4ii; lane E and G). The probe B3 did not detect P virus in any of the samples tested (Fig 3.4iii). E2a produced the strong signals with low background and was selected for further testing. No signals were detected on membranes without probe.

3.3.3 DNA sequence analysis and probe production by PCR

DNA sequence analysis of E2a revealed a 52 bp poly A region at the 3' end of a 712 bp sequence. Forward (F1) and reverse (R1) primers were designed to amplify a 527 bp region of the sequence, excluding the poly A tail by PCR (Fig 3.5). Electrophoresis of the PCR product showed a discrete band at a position of approximately 500 bp, indicating successful amplification of the fragment (Fig. 3.6). This product was successfully labelled and designated E2b.

3.3.4 Detection of virus with E2b probe by dot blot hybridisation.

The probe E2b (produced by PCR) was tested for specificity to P virus by dot blotting. It produced positive signals with positive controls (i.e. pUC18 containing the E2 insert, see lane A and P). It was specific for P virus, producing a signal with a pure sample of P virus (lane B). It also produced positive signals with tissue homogenates of animals known to be infected with P virus (lanes G, H, I, L, and O). No reaction was observed with uninfected *L. depurator* tissue (D, J, K, M and N), purified W2 virus (lane C) or healthy C. *maenas* tissue (lane E and F) (Fig. 3.7).

3.3.5 *In situ* hybridisation

The probe E2b produced a positive reaction in P infected *L. depurator,* indicated by strong staining in the connective tissue of hepatopancreas (Fig 3.8A). No reaction was observed in the same tissues subjected to the *in situ* hybridisation procedure without probe or without anti-DIG AP. (Fig 3.8B). Further experiments using E2b to probe tissue sections from healthy *L. depurator* and C. *maenas* are described in Chapter 4.

3.4. DISCUSSION

This chapter reports the construction of the first specific gene probe (E2b) to a virus which infects a temperate water marine decapod. The probe successfully detects P virus in tissue homogenates and paraffin embedded sections of *L. depurator* tissues known to be infected with P virus. Importantly, it does not give positive reactions with P - negative tissues from *L. depurator* nor a closely related crab species, *C. maenas*. DNA sequence analysis of the gene probe allowed primers to be designed to amplify the probe by PCR. This enabled large quantities of probe to be produced reliably and more rapidly than more traditional cloning methods.

E2a was initially produced by digestion using the enzymes Kpn I and Bam HI to remove the insert from pUC18 plasmid vector. This probe produced strong signals and low background, although it also exhibited non-specific reaction with P - negative tissues. There are a number of possible explanations for non-specific binding involving different stages of the hybridisation protocol including denaturation, hybridisation and washing (Leitch *et al.*, 1994). It is unlikely, in this case, that non-specific binding was due to the protocol because stringency was increased from 60 ± 5 % to 88 ± 5% by inclusion of a washing step at 68° C. This denatured and removed weakly bound probe. Another possible explanation is that the affinity of a probe for target nucleic acid depends primarily on its internal sequence (Bruce *et al.*, 1993). In the present study, DNA sequence analysis of the E2a sequence revealed a poly A region at the 3' end of the probe. Since the probe is double stranded, there is a corresponding poly T region which binds to the polyadenylated region of messenger RNA found in all cells. When E2 was amplified without the poly A region by PCR and labelled to make a probe (E2b), specific reactions were observed

consistently in both homogenates and sections of P-positive tissues. Therefore it appears the non-specific reactions were due to the internal sequence of the probe.

Of the two other probes tested, D6 and B3, D6 produced similar results to E2a although the signals were much weaker. By contrast, B3 failed to produce any signals with target RNA in tissues, although it hybridised strongly with the positive control, i.e. pUC18 containing the B3 insert. Since the probe bound to a positive control in conditions of low stringency (60 ± 5 %), it would appear that the hybridisation conditions were satisfactory and that the most likely cause is inefficient binding due to degradation of the target (Leitch *et al.*, 1994). The probe B3 corresponds to the small segments of the genome which are all less than 1.2 kbp in length (Montanie *et al.*, 1993). At 350 bp, B3 was also the smallest probe tested.

In the present study, attention was focused on producing one highly specific probe for P virus, but it is apparent that sequencing the other two probes, B3 and D6, would also enable specific regions of these fragments to be amplified and may increase their specificity. Clearly, three probes, corresponding to different regions of the genome would increase the possibility of detecting virus in infected animals.

There are numerous potential applications for gene probes which hybridise specifically to viral nucleic acids in infected animals. They are important diagnostic tools and a number have now been developed to detect shrimp pathogens such as BP (Bruce *et al.*, 1993; Lu *et al.*, 1993; Bruce *et al.*, 1994; Bonami *et al.*, 1995; Nunan *et al.*, 1997 and Durand *et al.*, 1998), HPV (Mari *et al.*, 1995), IHHN (Carr *et al.*, 1996) and TSV (Mari *et al.*, 1998). Gene probes can also be used to localise the sites of viral infection at the cellular level by *in situ* hybridisation (Durand *et al.*, 1996;

Bruce *et al.*, 1994) and to track the lytic cycle of virus inside host cells. The detection of P virus in connective tissues of hepatopancreas using E2b corroborates findings of Bonami *et al.*, (1976) that P virus primarily infects this tissue. This probe could be used to determine other sites of infection in crabs, and may provide important information on the transmission routes of this virus.

The construction of probes by PCR may allow the development of *in situ* PCR to detect crustacean viruses. This new technique has recently been used in human and veterinary medicine (Walker *et al.*, 1995). *In situ* PCR combines the sensitivity of PCR reaction to intracellular localisation of genomic sequences and has the same specificity as *in situ* hybridisation (Walker *et al.*, 1995). Target nucleic acid sequences can be amplified by PCR then detected by *in situ* hybridisation (Walker *et al.*, 1995). Obviously, such technology could have important implication in diagnosis of viral diseases in crustaceans, particularly in diagnosing latent infections.

Certainly, advances have been made in the rapid diagnosis of viral diseases from shellfish using gene probes, however, there has been little attempt to use such technology to investigate virus-host interactions at the cellular and population levels in invertebrates. This is primarily because of the lack of suitable model systems and *in vitro* cell cultures. It is essential to develop model systems to investigate viral disease in crustaceans because they provide the opportunity to investigate virus host interactions without constraints, such as containment of pathogens, that affect this commercially important invertebrate group.

Figure 3.1 An outline of the main procedures in gene probe construction.

cDNA is synthesised from segments of the P virus genome. Three fragments of cDNA are selected for use as gene probes, D6, E2a and B3. These are ligated into pUC18 plasmid vectors to form recombinant plasmids. The plasmids are transformed in *E. coli* (DH5-α) and cloned. Plasmids are purified from bacterial hosts using minipreparation kits and the cDNA inserts extracted with restriction enzymes. The digestion products are visualised on 1 % agarose gels and extracted and purified using spin columns. The purified cDNA inserts are labelled using the random primed labelling method to produce digoxigenin labelled probe.

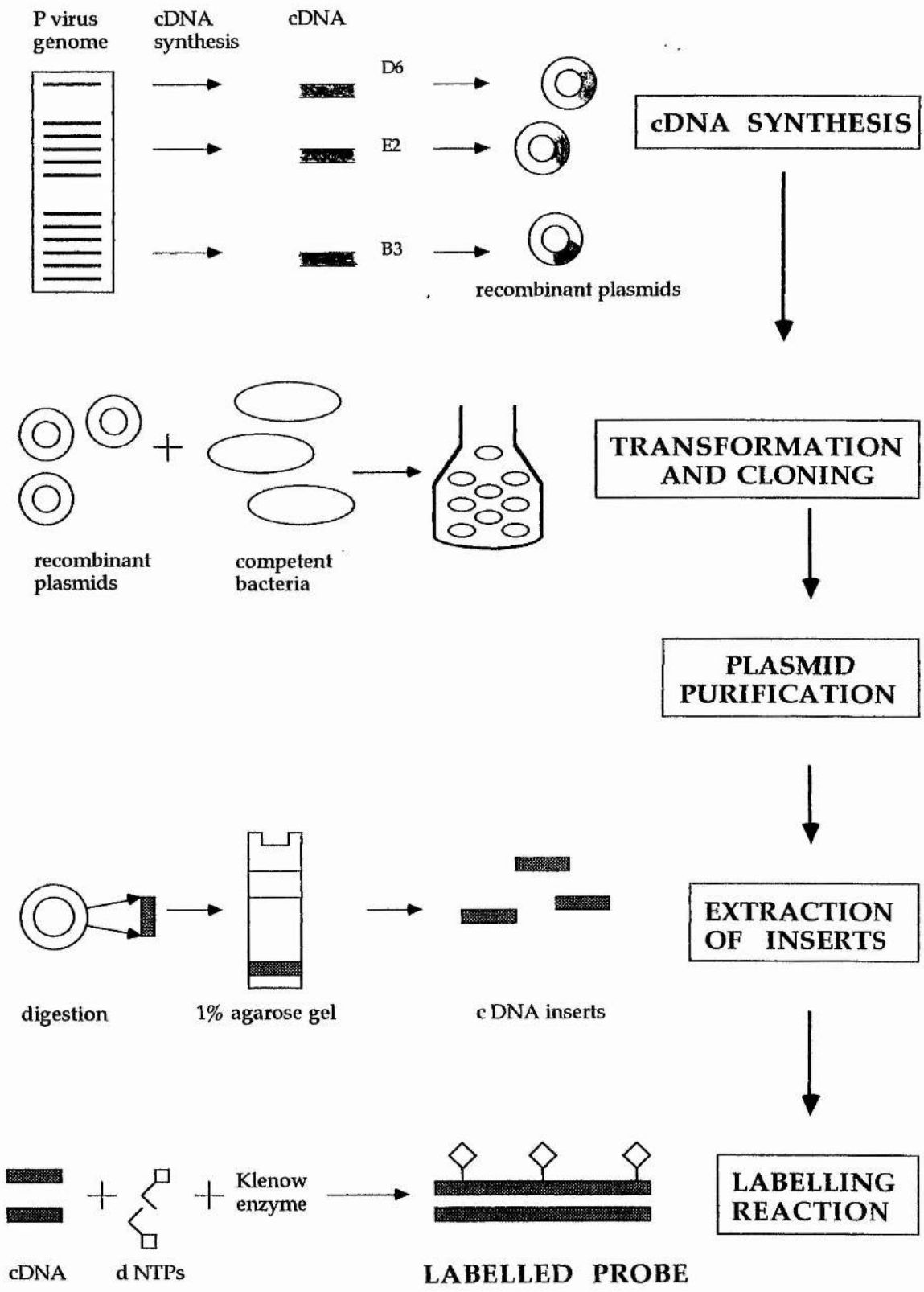

P virus genome
cDNA synthesis
cDNA
D6
E2
B3
recombinant plasmids
cDNA SYNTHESIS
TRANSFORMATION AND CLONING
recombinant plasmids
competent bacteria
PLASMID PURIFICATION
EXTRACTION OF INSERTS
digestion
1% agarose gel
c DNA inserts
LABELLING REACTION
Klenow enzyme
cDNA
d NTPs
LABELLED PROBE

Figure 3.2 An outline of the major steps for *in situ* hybridisation.

PREPARATION

Tissues are fixed in formaldehyde and embedded in paraffin.

↓

DENATURE PROBE AND MATERIAL

RNA (tissue) and DNA (probe) are denatured at 65° C and 100° C respectively for 10 min then placed on ice.

↓

HYBRIDISATION

Labelled probe is added to high SDS buffer.

↓

WASHING

Weakly bound probe is removed by washing in detergent.

↓

DETECTION

Colormetric detection of anti-DIG-AP with the substrates NBT/X-phosphate.

Figure 3.3 Products of the digestion of plasmid (pUC 18 containing E2a, D6 or B3). Samples were run on 1 % agarose gel containing 0.5 μg ml^{-1} ethidium bromide (final concentration).

D6 = digestion of pUC18 containing D6 fragment with Eco RI (fragment is approx. 500 bp).
E2a = digestion of pUC18 containing E2a fragment with KpnI and BamHI (fragment is approx. 700 bp).
B3 = digestion of pUC18 containing B3 fragment with KpnI (fragment is approx. 700 bp).
A = 1kb DNA ladder (Gibco BRL).
B = λ DNA digested with Eco RI.
C = λ DNA digested with Hind III and Eco RI.

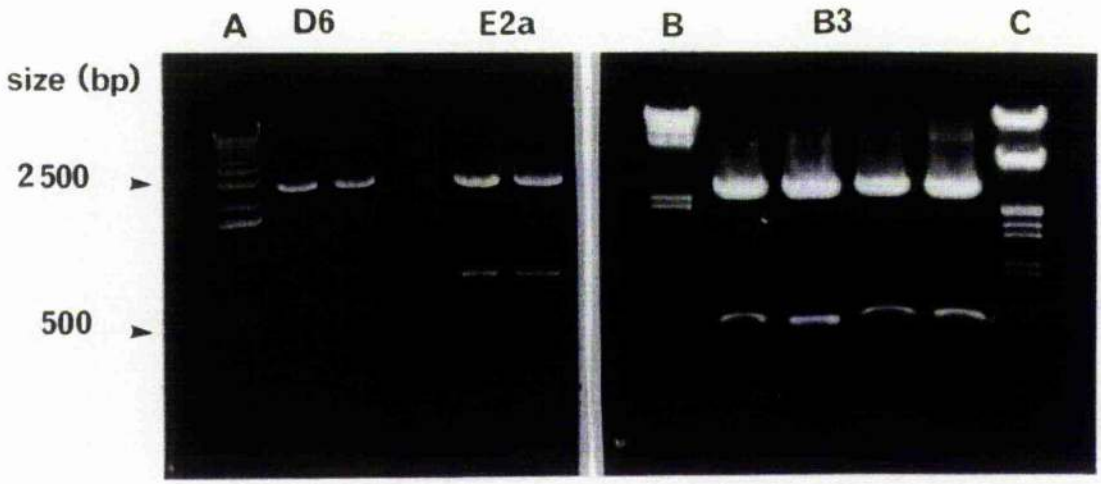

A
D6
E2a
B
B3
C
size (bp)
2 500
500

Figure 3.4 Testing the gene probes E2a, D6 and B3 by dot blot hybridisation. 1 μl samples are dotted onto nylon membranes. The probes hybridise to viral RNA in the samples and positive signals are revealed by colourmetric detection. Positive signals appear as dark dots on the membrane.

(i) Dot blot results using E2a probe.

(ii) Dot blot results using D6 probe.

(iii) Dot blot results using B3 probe.

A = positive control (plasmid containing E2a, D6 or B3)

B = purified sample of P virus

C = purified sample of W2 virus

D = homogenate of P infected tissue from *L. depurator*

E and G = homogenates of tissue from healthy *C. maenas*

F = homogenate of tissue from healthy *L. depurator*

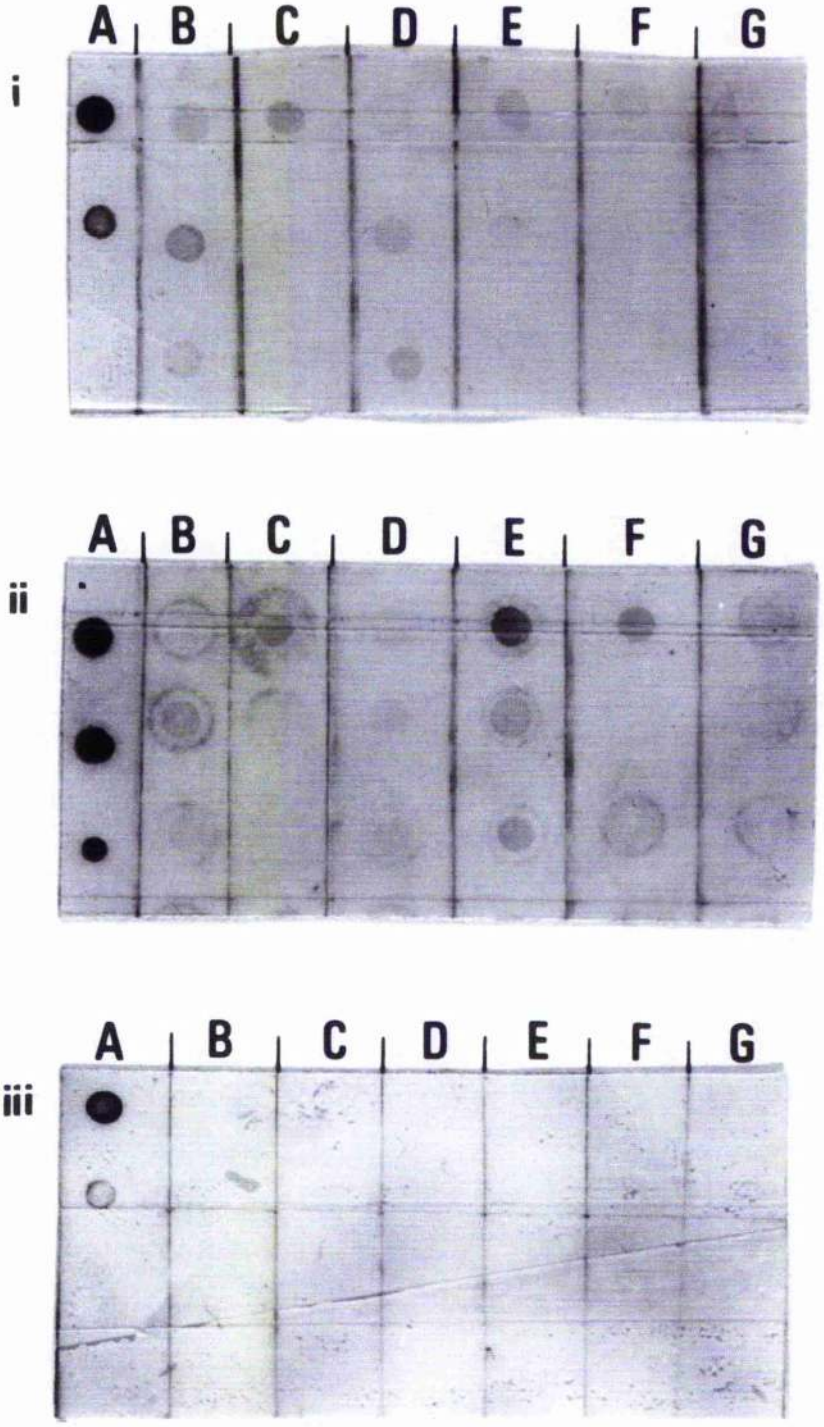

A B C D E F G
i
A B C D E F G
ii
A B C D E F G
iii

Figure 3.5 DNA sequence of the E2a fragment (712 bp). A 52 bp poly A tail exists at the 3' end of the sequence (bases 661-712). The region in **boldtype**, is the sequence of the primer F1, 5'CAG ATG CTG ATG TTT CAG AG3' and the region underlined is that to which the reverse primer R1, 5'CCA GCA AGT GTC TAA ACA TA3' anneals. These two primers are used to amplify a 527 bp region of the E2a sequence. This region is designated E2b.

5' 40
CGGAGAAGCA AATCCGAAAA GAAAGAGAGA ATGTT**CAGAT**

80
GCTGATGTTT CAGAGAGGGA TACCTCAACA ACCTGACGAA

130
TATTGTACTC TGGCATCTGA ATCGGACCAC CTGTTATACA

170
CCGTTCATTC CCTTGGAGCC AGGGCAGCTA TTTGTAGCAT

210
ACAGAACTTG TTGTACTATC CGTTGAGTGT CGATGCACCT

250
TTCAGATTGA GAGGATTGGT GACATATTTA ACGGACTCTA

280
AATTTTAGCA ATGAGACTAA TGTGATAGGA ACACTCCAGA

330
ACCTGAAGGA TCATCCATTA TTCAATGAAT ACATCAGTCC

370
TCGCTGGGAC AAGATTAAAG CCTTTCTTAT GATGATGAGT

410
GCGTCAGGGA AGCCCCAGTC TGTTTCAGAT GATTATGAAG

450
ATTATTTGAC TGATGACCGG ATAACGACCG TGACTTTCAA

490
AGGATTTACC GTAGTCCACG CCATTTTTTC GGTAAGGTAT

530
GGTAACAGCG AGATGGAGGG AGAGTTTTAC ACAATTAGTG

570
CGACCTCCGC TGATATGTTT AGACACTTGC TGGCCAACTA

610
CCACTATTCA GAGGGCGCGT GAGGGATGCA GGACGCCACA

650
CTTTCGGTTT GAACTGAATT ACTGCTGGCG CAGGCACTGA

680
GATCAACGAT AAAAAAAAAA AAAAAAAAAA

710 3'
AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AA

Figure 3.6 Amplification of E2b by PCR. A = 5 samples of pUC18 plasmid containing E2a insert, primers and 'Ready to Go' beads. M = molecular weight marker (1 kb DNA ladder). E2b is amplified from E2a, without the poly A region, because primers are designed to exclude the poly A region (see Fig. 3.5). E2b is approximately 500 bp.

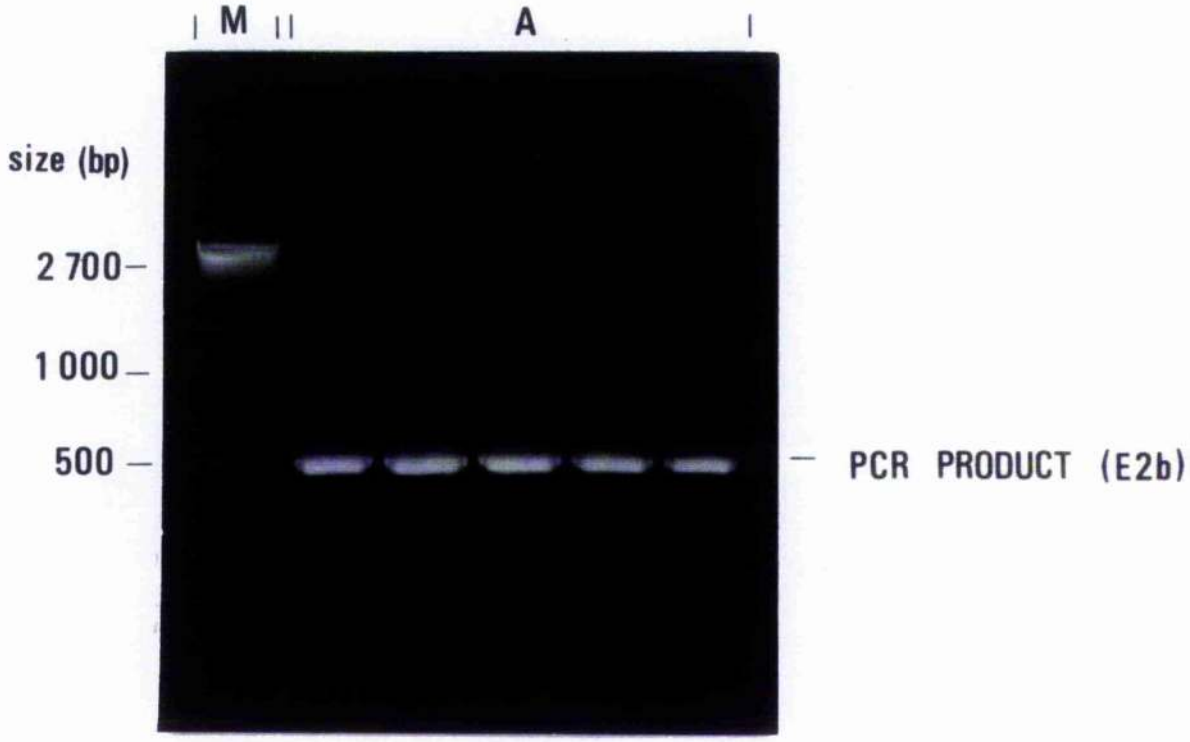

M
A
size (bp)
2 700
1 000
500
PCR PRODUCT (E2b)

Figure 3.7 Testing the E2b probe using dot blot hybridisation. Dots on the membrane indicate P virus is present in the sample. 1 μl samples are dotted onto nylon membranes. The probes hybridise to viral RNA in the samples and positive signals are revealed by colourmetric detection. Positive signals appear as dark dots on the membrane.

Variation in intensity of dots between samples is probably due to differences in virus numbers in each sample.

A = positive control (plasmid containing E2 insert);

B = purified P virus;

C = purified W2 virus;

D, J, K, M, N = healthy *L. depurator* tissue;

E, F = healthy *C. maenas* tissues.

G, H, I, L, O = P-infected *L. depurator* tissues.

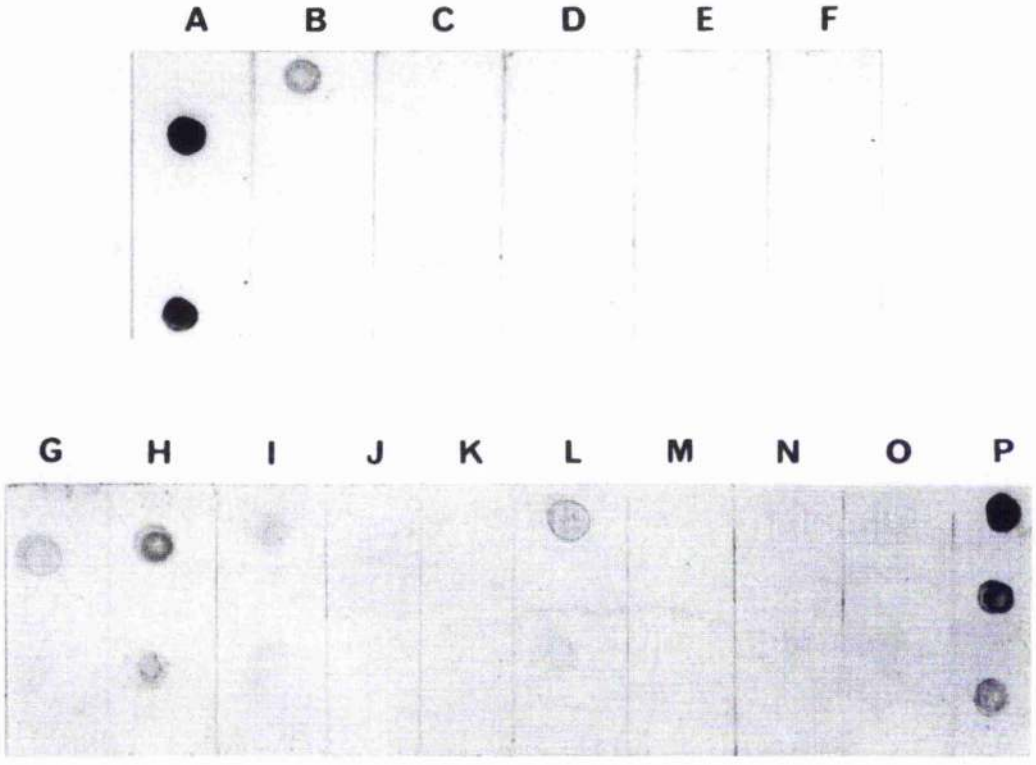
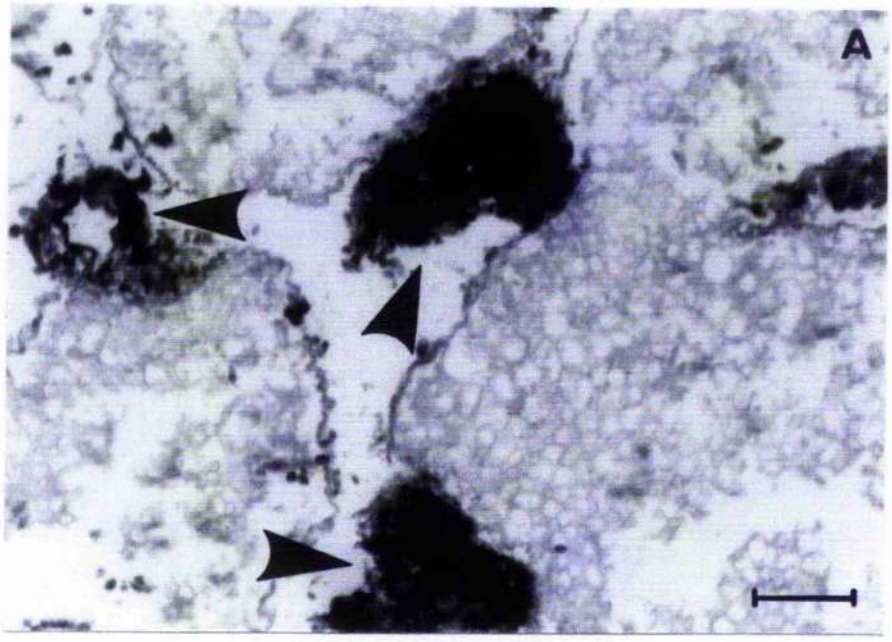
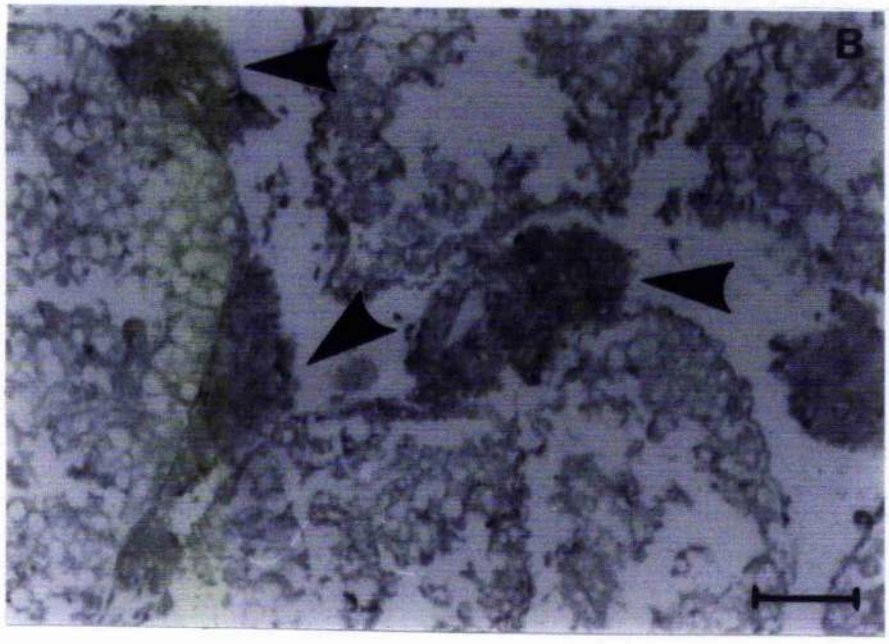

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P

Figure 3.8 *In situ* hybridisation on paraffin embedded hepatopancreas of *L. depurator* known to be infected with P virus. The gene probe E2b was used to detect P virus in tissues.

(A) P infected hepatopancreas from *L. depurator*. Positive signals (arrows) can be seen in the connective tissues of the hepatopancreas. No signals are evident in the tubules of the hepatopancreas. Scale bar = 25.0 μm

(B) Hepatopancreas from the same P infected *L. depurator* used in A. Tissue on this slide served as a negative control because no probe was used in the hybridisation protocol. No signals are evident in either connective tissue or hepatopancreatic tubules. Scale bar = 25.0 μm.


A


B

Chapter 4

USE OF THE E2b GENE PROBE TO STUDY P VIRUS INFECTIONS IN MARINE DECAPODS

4.1 INTRODUCTION

The effective management of viral diseases in the aquaculture industry has been severely hampered by the lack of rapid diagnostic procedures for the identification of viral pathogens. However, recent advances in molecular biology have lead to the development of gene probes, now recognised as amongst the most sensitive and accurate ways to detect viruses in animals tissues.

Gene probes are labelled fragments of DNA or RNA used to detect and identify specific nucleic acid sequences by selectively hybridising to them. The first report of the use of gene probes dates back to 1969 when Pardue & Gall (1969) visualised the genes encoding ribosomal RNA in toad, Xenopus *laevis* oocytes by *in situ* hybridisation using radioactively labelled probes. The first use of gene probes in virology followed a year later, in 1970, when Orth *et al.* visualised Shope rabbit papilloma virus genomes in tumours. Since then, improvements in probes and techniques has lead to an increase in the diversity of uses for gene probes in virology. These include elucidation of the mode of viral replication, identification of modes of transmission and dissemination, localisation of sites of active viral infection, development of viral infectivity assays and disease diagnosis reviewed by Teo (1990).

Gene probes have recently been used to study viral infections in crustaceans. The first successful application was the detection of shrimp baculovirus in fixed tissues from penaeid shrimps (Bruce *et al.*, 1993). Since then, construction of gene probes to other viruses has focused on those from shrimps because they are a commercially important species. These probes have been used for a number of applications including disease diagnosis (Carr *et al.*, 1996; Mari *et al.*, 1998); localising sites of infection within tissues (Bruce *et al.*, 1994; Mari et al., 1995; Durand *et al.*,

1996) and assessing virulence of pathogens from different geographical locations (Bruce *et al.*, 1993; Durand *et al.*, 1998). As yet, gene probes have not been used to study the prevalence and ecology of crustacean viruses from wild populations.

Improvements in techniques have resulted in gene probes being constructed by PCR (Lu *et al.*, 1993; Nunan *et al.*, 1997), a technique which enables large quantities of probe to be produced reliably and rapidly. The construction and development of a gene probe for P virus using PCR is described in Chapter 3. The probe has a great deal of potential because it offers the opportunity to study a viral infection in a model system without the constraints and containment problems associated with investigating commercially important, intensively reared stocks; such as penaeid shrimps.

The aim of this chapter is to use the gene probe developed for P virus to (a) investigate pathogenicity of P virus at different times of year in experimentally infected and control *L. depurator*, (b) assess the specificity of P virus for this host and (c) to localise sites of infection within tissues of susceptible crabs.

4.2 MATERIALS AND METHODS

4.2.1 Animals

L. depurator and C. *maenas* were collected and maintained as described in Section 2.2.1.

4.2.2 Virus propagation

To investigate seasonally related changes in disease development and changes with passaging, experiments were undertaken at various times of the year. The first passage of P virus in North Sea *L. depurator*, using a homogenate of gills and hepatopancreas from infected Mediterranean crabs (named P1) is described in chapter 2 (section 2.2.2). The hepatopancreas and gills of an animal that developed P virus infection during the first passage were homogenised, as described in 2.2.2, named P2 and injected into three batches of 8 - 10 *L. depurator* in March, June and July 1997, as described in 2.2.2. Animals were maintained for up to 30 days, as described in section 2.2.2, at sea water temperatures of 7, 13 and 16° C during March, June and July respectively. Hepatopancreas and gills of three animals that developed P virus during the second passage were homogenised, designated P3a, P3b and P3c and used to inoculate batches of 10 - 15 *L. depurator* in November 1997, January, May and June 1998. Animals were maintained as described in section 2.2.2 at sea water temperatures of 8, 5, 10, and 13° C in November 97, January, May and June 98 respectively. For each experiment, equal numbers of control animals were given injections of sterile TN buffer (Appendix 1), as described in 2.2.2. The time to death was recorded (in days) and hepatopancreas and gills were removed from all animals at death. Animals remaining alive after 30 days were sacrificed and hepatopancreas and gills also excised. Approximately half the tissues excised from each animal were frozen at -20° C and half fixed in modified Davisons fixative (Hasson *et al.*, 1997; Appendix 1) for 24 h, rinsed in 70 % alcohol, then stored in 70 % alcohol until required.

To confirm that P virus does not infect the closely related crab species, C. *maenas;* batches of 5 - 10 C. *maenas* were inoculated with

samples P1, P2, and P3b and P3c in parallel to experiments described in 4.2.2. For each animal 0.3 ml of sample was used for inoculation via the base of the fifth pereiopod. P1 was used to inoculate C. *maenas* in October 1996, P2 in June and July 1997 and P3b and P3c in May and June 1998 respectively. Control C. *maenas* were inoculated with sterile TN buffer (Appendix 1) as described in section 2.2.4. Animals were maintained as described in 2.2.4. Tissues from all animals that either died within 30 days or that were sacrificed after 30 days were processed as described above.

4.2.3 Probe construction

The probe E2b was prepared for the P virus genome as described in section 3.2.6.

4.2.4 Dot blot hybridisation

To determine the prevalence of P virus infection in experimentally or buffer inoculated *L. depurator* and C. *maenas* throughout the year, frozen tissue samples obtained from propagation experiments described in section 4.2.2 were screened by dot blot hybridisation. Tissue samples from each animals was homogenised and 1 μl drops were placed on nylon membranes for dot blot analysis following the procedure described in 3.2.3. Screening of samples revealed host mortality levels from P virus in each treatment group at different times of the year, defined as the percentage infection. The time from inoculation to first death from P virus, is referred to as incubation time, expressed in days.

4.2.5 *In situ* hybridisation

To localise the sites of viral infection within tissues, *in situ* hybridisation was performed on sections of hepatopancreas from *C. maenas* or *L. depurator* using the method described in 3.2.4.

4.2.6 Statistics

Product moment correlation coefficients *(r)* were determined to examine the relationships between temperature and incubation time or temperature and % infection. The significance of the correlations was determined by using tables prepared for the critical values of *r* at (N - 2) degrees of freedom (Parker 1979). Accepted level of significance was $P < 0.05$.

4.3 RESULTS

4.3.1 Dot blot hybridisation

Screening of hepatopancreas and gill homogenates of both species by dot blot hybridisation revealed which *L. depurator* or *C. maenas* had detectable levels of P virus in their tissues at the time of death, or at time of sacrifice (30 days post inoculation), by the appearance of dots on the nylon membrane. Positive signals were observed in both P inoculated and control *L. depurator* (Fig 4.1).

The percentage of animals that developed P virus infections and the incubation time from inoculation to the first detection of P virus in tissues, are shown in Table 4.1. *L. depurator* inoculated with homogenates from other North Sea *L. depurator* (P-treated) showed reduced incubation times from 21 days at 4.7° C to 4 at 15.8° C days (Table

4.1). Interestingly, a significant correlation was observed between incubation time and sea water temperature in the P-treated animals, incubation time decreased as sea water temperature increased ($r = 0.93$, $P < 0.05$; Fig 4.2). Results from October 1996 were excluded from the analysis because these crabs were inoculated with a homogenate prepared from Mediterranean crabs.

Mortality in P - treated *L. depurator* ranged from 60 to 100 % within 30 days of inoculation although the percentage of mortality did not reflect the numbers of animals that developed P virus infection (Table 4.1). The percentage of crabs that were infected with P virus ranged from 12.5 - 75.0 %. There was significant correlation between the percentage infection and sea water temperature in *L. depurator* inoculated with homogenates from other North Sea crabs, percentage of infection increased with increasing sea water temperature ($r = 0.79$, $P < 0.05$, Fig 4.3).

Passage of P virus resulted in a decrease in incubation time and increase in percentage infection (Table 4.2). Only passages performed at the same sea water temperature were compared because there is significant correlation between percentage infection and sea water temperature, described above. In the first passage (P1), using P virus from Mediterranean *L. depurator*, P virus was not detected in tissues until 36 days post inoculation. At the second (P2) and third passage (P3) P virus could be detected after only 3 days (Table 4.2). The percentage of infection increased with successive passages from 12.5 % at P1 to 55.5 % at P2 and 73.0 % at P3 (Table 4.2). One experiment was completed for each passage, therefore, it is not possible to determine whether the observed increase in percentage infection is statistically significant.

As with P-treated *L. depurator*, dot blot hybridisation revealed the percentage of animals that developed P virus infections and the incubation time of P virus in control *L. depurator* (Table 4.3). In control, buffer treated *L. depurator*,, incubation time ranged from around 30 days at 7.0° C to 3 days at 13.0° C and was significantly correlated to sea water temperature, incubation time decreased with increasing temperature ($r = 0.85$, $P<0.05$) (Fig 4.4).

Mortality in control *L. depurator* ranged from 40 to 100 % within 30 days of inoculation although, as with P treated crabs, the percentage of mortality did not reflect the numbers of animals that developed P virus infection (Table 4.3). The percentage of animals that were infected with P virus ranged from 0.0 % at 4.7° C to 100.0 % at 15.8° C. This correlation between percentage infection and sea water temperature was significant, percentage of infection increased with increasing sea water temperature ($r = 0.86$, $P < 0.05$) (Fig 4.5).

4.3.2 Effect of P virus on *C. maenas*

In *C. maenas* treated with homogenates containing P virus or with TN buffer, mortality ranged from 0.0 - 45.5 % (Table 4.4). However, dot blot analysis (Fig 4.6) indicate that none of the crabs had detectable levels of P virus in their tissues. Thus P virus seems to fail to establish infection in this species of crab (Table 4.4).

4.3.3 *In situ* hybridisation.

In situ hybridisation of paraffin embedded sections from *L. depurator* revealed sites of infection within the hepatopancreas (Fig 4.7A). The connective tissues were heavily stained, indicating the presence of P virus. Regions of necrosis were also clearly visible in

infected tissues (Fig 4.7A). The tubules and epithelial tissues remained unstained, indicating the absence of virus (Fig 4.7A). Tissues from *L. depurator* which had not died from P virus infection did not show any staining in tissues from the hepatopancreas (Fig 4.7B).

The hepatopancreas from *C. maenas* showed no evidence of P virus infection (Fig. 4.7C). Connective tissues from *C. maenas* inoculated with P virus (Fig. 4.7C) and those inoculated with buffer (Fig. 4.7D) also showed no signs of staining, confirming the absence of P virus in the tissues of this species.

4.4 DISCUSSION

This chapter reports the first use of a gene probe to investigate a pathogen of temperate water crustaceans. P virus infections were detected in both P-treated and control *L. depurator*. The detection of P virus in control *L. depurator* indicates that P virus is prevalent in wild populations from the North Sea. Although the animals were treated with TN buffer, it is highly unlikely they contracted P virus in the aquarium because they were isolated from P treated animals. However, screening of untreated *L. depurator* from wild populations is necessary to confirm these findings.

For both P-treated and control *L. depurator*, P virus infection was influenced by temperature. There were seasonal variations in both the percentage of animals with infection, which positively correlates with sea water temperature, and in the incubation time of virus infection, which negatively correlates with sea water temperature. These findings indicate that *L. depurator* is more susceptible to P infection at high sea water temperatures. Interestingly, Chisholm & Smith (1994) reported

that for the shore crab, *C. maenas* there is a reduction in both haemocyte number and antibacterial activity at extremes of sea water temperature, notably at approximately 15° C. It is apparent that there is a reduction of immunocompetence at extreme high or low sea water temperatures, although it is difficult to determine why this should occur because of the numerous intrinsic and extrinsic factors which affect haemocyte number and, consequently, immunocompetence. The pathogen itself, may also be affected by temperature; indeed reoviruses from mammals are inactivated at high temperatures by the removal of σ-1 attachment protein (Drayna and Fields 1982). At low temperatures, it has been found that prolonged storage of reoviruses at 4° C could lead to a loss of infectivity (Tyler and Fields 1996). In this study little or no P infections were found in January when the sea water temperature ranges from 3 - 5° C. Therefore, it is likely that the high incidence of P infections in *L. depurator* during the summer months is due to a combination of immunosuppression and increased viral infectivity.

With P-treated *L. depurator*, mortality levels during each experiment did not reflect the incidence of P infection. This is not surprising given that each animal was injected with a homogenate of tissue from another animal resulting in numerous non-self signals that could activate the host immune system. It is also possible that individuals may have had other infections even though they were quarantined for at least one week prior to inoculation. In the P-treated *L. depurator*, it is apparent that passaging of P virus results in an increase in mortality and, importantly, a dramatic increase in P infection and decrease in the incubation time. Although equal amounts of tissue and buffer volumes were used in the preparation of samples for inoculation and passaging, the number of infectious particles was unknown because

it is impossible to standardise the number of infectious units due to the lack of suitable techniques in invertebrate virology. It is therefore unclear whether the increase in incidence due to passaging was caused by an increase in the numbers of infectious particles in each sample or a mutation in the virus that increased infectivity.

In Chapter 2, analysis of *C. maenas* tissues under TEM provided preliminary evidence that P virus does not infect shore crabs. Dot blotting of P-treated and control *C. maenas* tissues using E2b probe confirmed these findings. The lack of susceptibility of *C. maenas* to P virus infection is unknown but it is likely to involve inability of P virus to bind to receptors on the surface of *C. maenas* cells. This is the first stage in the reovirus replication without which infection cannot be established (see review by Nathanson 1997). Thus, this phenomenon may prove to be an important aspect of disease pathogenesis for the investigation of defence responses to viral pathogens.

In the present study, *in situ* hybridisation was used to localise sites of viral infection within tissues. Tissue sections from the hepatopancreas of crabs were examined for P virus because this organ is known to be one of the main sites of infection (Bonami 1973). *In situ* hybridisation confirmed that P virus infects the connective tissues of *L. depurator*, but not the tubules or epithelial cells. Furthermore, no signals were detected in any tissues from *C. maenas*, providing further evidence that P virus does not infect *C. maenas* from the North Sea. It is unclear whether paralysis, the main symptoms of P virus infection, is caused by P virus directly infecting nerve cells or by the degeneration of other tissues but since *in situ* hybridisation was successful in localising sites of infection within the hepatopancreas, the technique could be applied to other tissues. *In situ* hybridisation can also be used to determine the mode of

transmission of virus infections. Indeed, Bruce *et al.* (1994) used this technique to examine the reproductive organs of penaeid shrimps for the presence of baculovirus. The absence of any baculovirus in the tissues indicated that transmission was horizontal by the *per os* route rather than true vertical routes (Bruce *et al.*, 1994).

The development and use of a gene probe for P virus has revealed important information about P virus infections in crabs that could not be easily determined by the more traditional methods of electron microscopy. The results from this study represent the first use of a gene probe to screen large numbers of temperate water crustaceans. This application has a great deal of potential for future investigations including screening animals from wild populations, to assess prevalence of disease in different geographical locations, and to determine the effects of different environmental parameters on host susceptibility to disease.

Figure 4.1. Screening of *L. depurator* tissues by dot blot hybridisation using the probe E2b. 1 μl samples of tissue homogenates were dotted onto nylon membranes. The probe hybridises to viral RNA in samples and positive signals are revealed by colourmetric detection. Positive signals appear as dark dots on the membrane. Variation in signal intensity is probably due to differences in virus numbers in each sample.

Samples 204, 29, 79, 49, 94, 95, 175, 38, 138, 172, and 80 are from tissues of *L. depurator* treated with TN buffer. Positive signals can be observed in samples 29, 79, and 172.

Samples 208, 90, 40, 108, 16, 126, 170, 171 are from tissues of *L. depurator* treated with homogenates containing P virus. Positive signals can be observed in samples 208, 90, 44, 126 and also, weakly in 170 and 171.

E2 is a positive control, the plasmid PuC18 contains the E2a insert from which the probe is made.

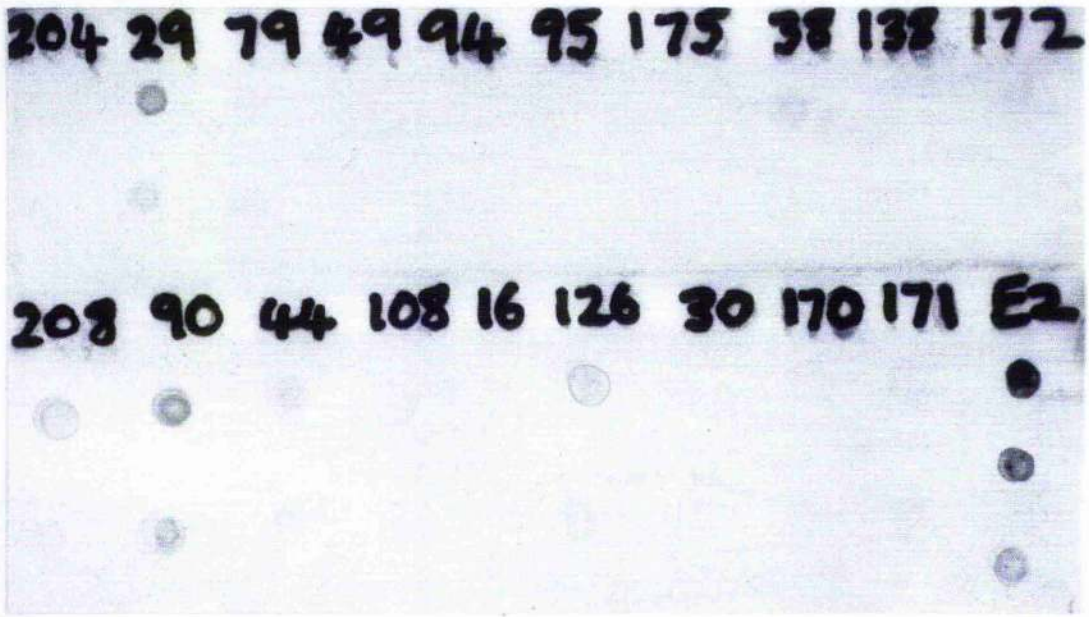

204 29 79 49 94 95 175 38 138 172
208 90 44 108 16 126 30 170 171 E2

Table 4.1. Experimental infection of *L. depurator* with P virus.

L. depurator were given inoculations of a homogenate containing P virus (see 4.2.2). Temperature is the mean sea water temperature of the holding tanks for each monthly experiment. Percentage mortality is the percentage of animals that died within 30 days of inoculation with P virus. Percentage infection is the percentage of all animals that contained detectable levels of P virus in their tissues. Incubation time is the time between inoculation and first detection of P virus. The total number of animals treated and screened for each experiment = n.

Table 4.1

Month/ Year	Temp. (°C)	Mortality (% ≤ 30 d)	P Infection (% of n)	Incubation time (days)	n
Oct 96	13	80	12	36	10
Mar 97	7	100	12	21	8
Jun 97	13	100	56	3	10
Jul 97	16	100	75	4	9
Nov 97	8	60	60	17	15
Jan 98	5	60	25	21	15
May 98	9	77	50	10	13
Jun 98	13	100	73	3	15

Figure 4.2 Correlation between incubation time and seawater temperature from *L. depurator* treated with P virus. The equation of the line of best fit is $y = 32.7467 - 2.0683x$ and $r = 0.93$. The correlation is significant ($P < 0.05$; 4 degrees of freedom). Values are expressed as days and correspond to the interval between inoculation and death from P virus.

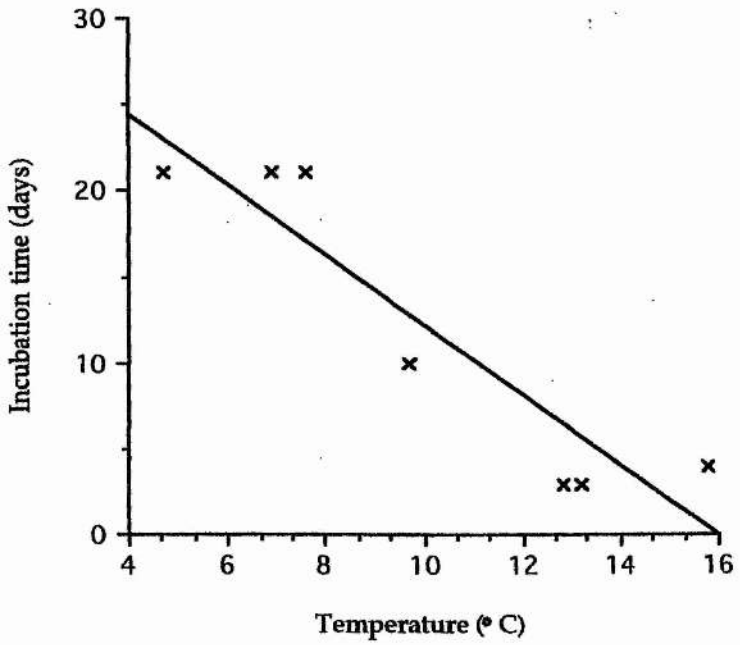

30
20
10
0
4
6
8
10
12
14
16
Incubation time (days)
Temperature (° C)

Figure 4.3 Correlation between percentage infection and seawater temperature from *L. depurator* experimentally inoculated with P virus. The equation of the line of best fit is $y = 2.2733 + 4.6618x$ and $r = 0.79$. The correlation is significant ($P < 0.05$, 4 degrees of freedom). Values are percentages from each monthly experiment.

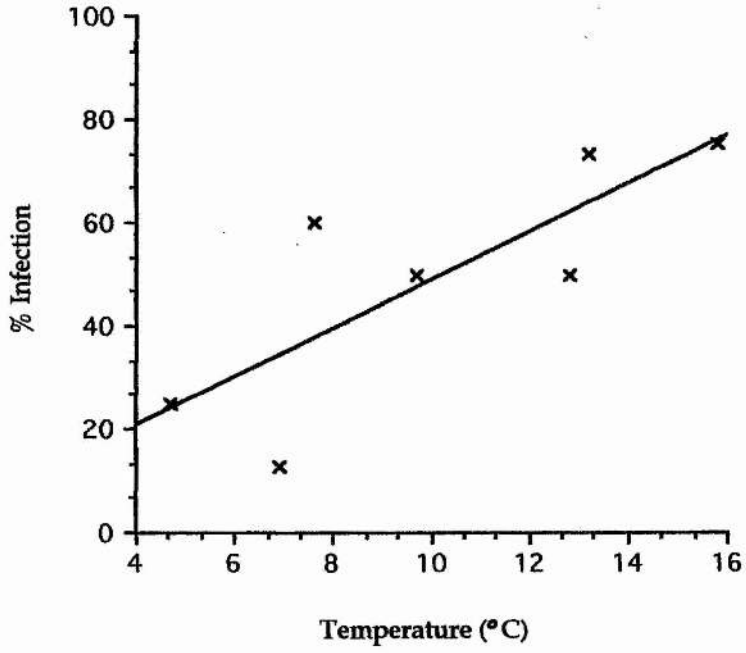

100
80
60
40
20
0
% Infection
4
6
8
10
12
14
16
Temperature (°C)

Table 4.2 Effect of passaging P virus in North Sea *L. depurator*. Passage 1 is experimental treatment of N. Sea *L. depurator* with homogenate from Mediterranean *L. depurator*, see 2.2.2. Passage 2 and 3 use tissue from other N. sea *L. depurator*, see 4.2.2. All other definitions are as described in Table 4.1.

Table 4.2

Temp (o C)	Month/ Year	Passage	Incubation time (days)	P infection (%)	n
7	Mar 97	1	21	12.5	10
7	Nov 97	2	17	60.0	10
13	Oct 96	1	13	12.5	10
13	Jun 97	2	12	55.5	10
13	Jun 98	3	7	73.0	15

Table 4.3 Prevalence of P virus in control *L. depurator*.
For definitions see Table 4.1.

Table 4.3

Month	Temp. (°C)	Mortality (% ≤ 30d)	P Infection (% of n)	Incubation time (days)	n
Oct 96	13	40	50	24	10
Mar 97	7	50	17	28	8
Jun 97	13	100	33	3	10
Jul 97	16	90	100	15	9
Nov 97	7	0	33	30	15
Jan 98	5	40	0	-	15
May 98	9	80	27	9	13
Jun 98	13	100	40	4	15

Figure 4.4 Correlation between incubation time and seawater temperature from swimming crabs treated with TN buffer. The equation of the line of best fit is $y = 40.5435 - 2.5646x$ and $r = 0.85$. The correlation is significant ($p < 0.05$; 3 degrees of freedom). Values are expressed as days and correspond to the interval between inoculation and death from P virus.

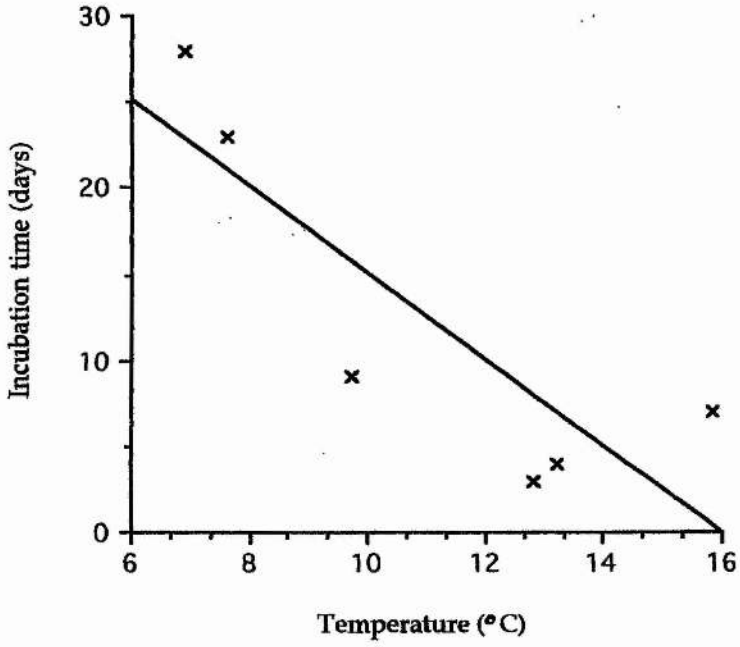

Incubation time (days)
30
20
10
0
6
8
10
12
14
16
Temperature (°C)

Figure 4.5 Correlation between percentage infection and seawater temperature from *L. depurator* treated with TN buffer. The equation of the line of best fit is $y = 6.8263x - 33.7881$ and $r = 0.86$. The correlation is significant ($P < 0.05$; 4 degrees of freedom). Values are percentages from each monthly experiment.

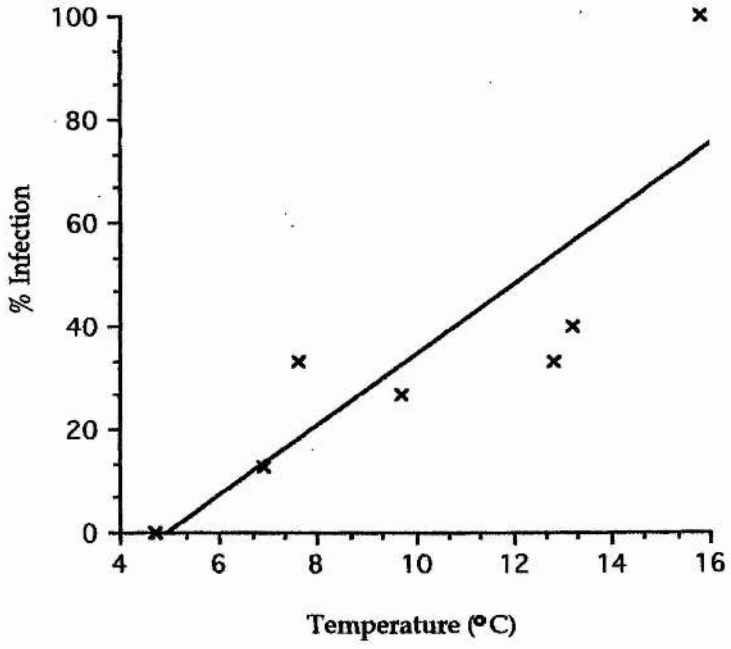

100
80
60
40
20
0
% Infection
4
6
8
10
12
14
16
Temperature (°C)

Figure 4.6 Screening of *C. maenas* tissues by dot blot hybridisation using the probe E2b. 1 μl samples of tissue homogenates wre dotted onto the nylon membranes. Probe hybridises to viral RNA in samples and positive signals are revealed by colormetric detection. Positive signals appear as dark dots on the membrane.

Samples 1, 2, 4, 5, 6, 8, 9, 13, 17, 19, 23, 25, 27, 28, 33, 36, 42, 43, 45, 47, 48, and 49 are from crabs inoculated with TN buffer. All appear to produce negative results (no dots).

Samples 3, 7, 10, 11, 12 14, 15, 16, 18, 20, 21, 22, 24, 26, 29, 30, 31, 32, 34, 35, 37, 38, 39, 40, 41, 42, 44, and 46 are from crabs inoculated with homogenates containing P virus. All appear to produce negative results (no dots).

Sample 50 is E2, a positive control consisiting of the plasmid PuC18 containing the E2a insert from which the probe is made.

The faint spots on samples 8 and 20 are probably dirt from the tissue homogenate.

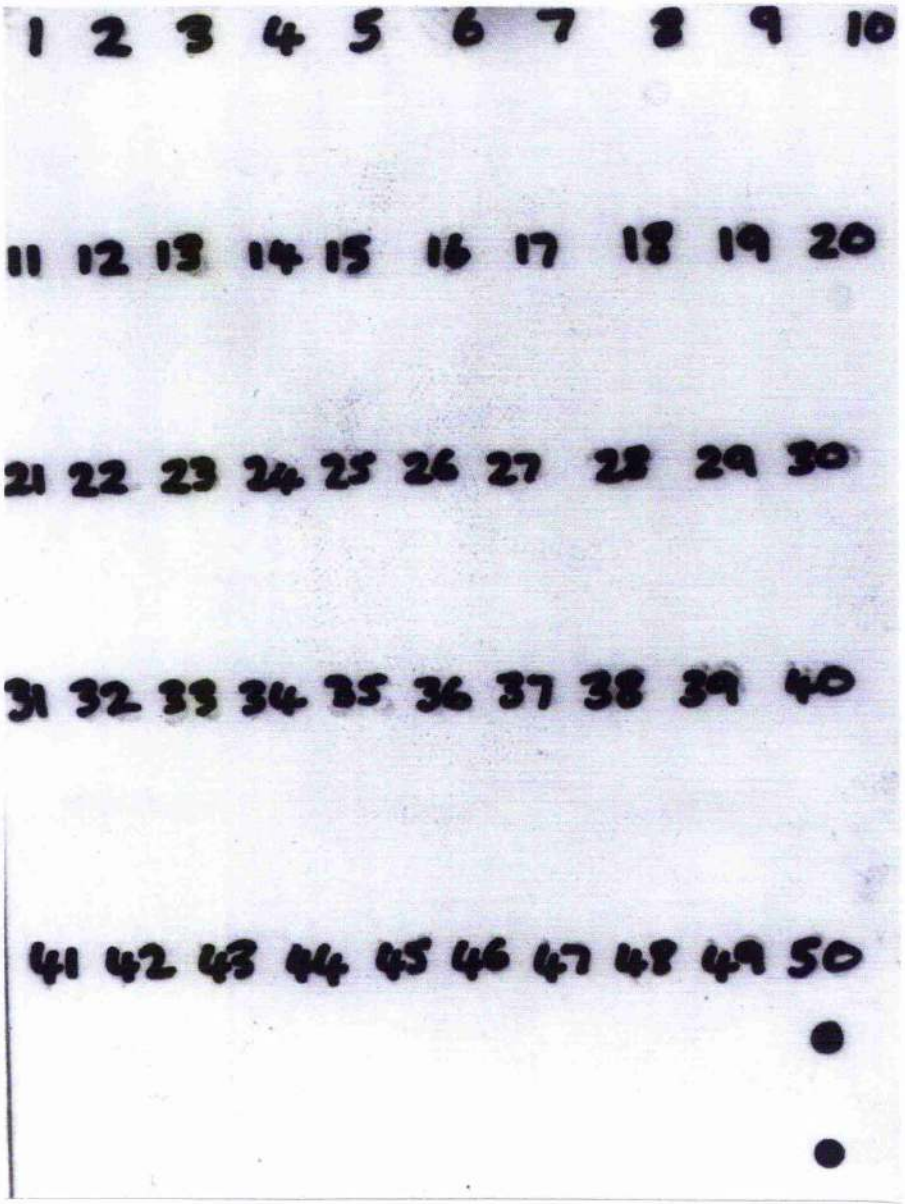

1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
21 22 23 24 25 26 27 28 29 30
31 32 33 34 35 36 37 38 39 40
41 42 43 44 45 46 47 48 49 50

Table 4.4 Experimental inoculation of *C. maenas* with P virus. Animals were given injections of P virus (P) or sterile TN buffer (TN), see 4.2.2. All other definitions are as described in Table 4.1.

Table 4.4

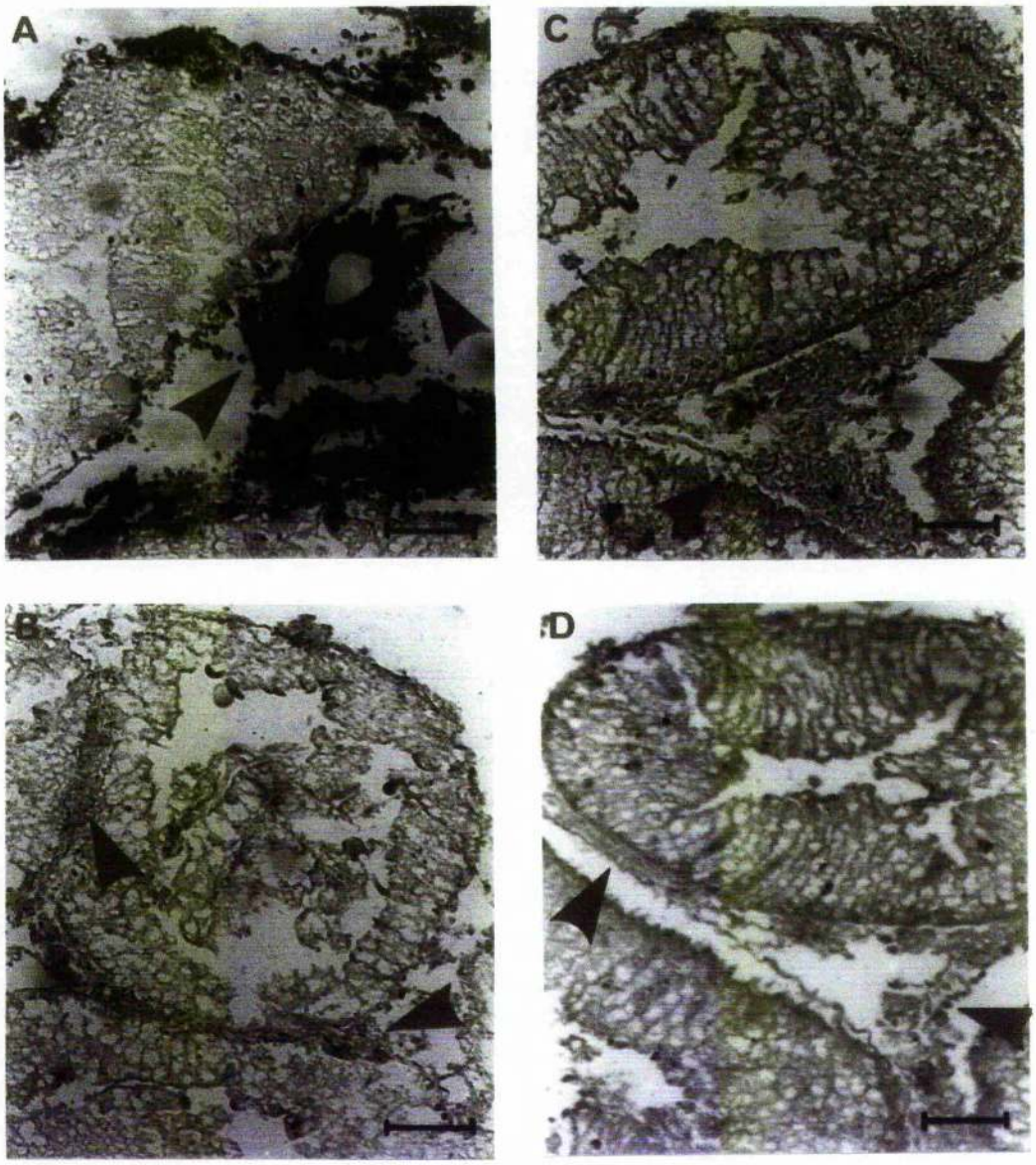
Month/ Year	Temp. (°C)	Treatment	Mortality (% ≤ 30 d)	P Infection (% of n)	n
Oct 96	13	P	20	0	10
		TN	**0**	**0**	**10**
Jun 97	13	P	83	0	6
		TN	**20**	**0**	**5**
Jul 97	16	P	0	0	10
		TN	**0**	**0**	**5**
May 98	9	P	46	0	10
		TN	**44**	**0**	**9**
Jun 98	13	P	43	0	7
		TN	**0**	**0**	**7**

Figure 4.7 A Tissue section of the hepatopancreas from *L. depurator*, infected with P virus. *In situ* hybridisation using the E2b probe revealed sites of viral infection within the tissue (arrows). Positive signals can be seen clearly in connective tissues. The tubules remain uninfected. Scale bar = 25 μm.

Figure 4.7 B Tissue section of the hepatopancreas from an uninfected *L. depurator*. *In situ* hybridisation using the E2b probe produced no signals within the tissues, indicating absence of P virus. Scale bar = 25 μm.

Figure 4.7 C Tissue section of the hepatopancreas of *C. maenas*, treated with P virus. *In situ* hybridisation using the E2b probe produced no signals within the tissues, indicating absence of P virus. Scale bar = 25 μm.

Figure 4.7 D Tissue sections of the hepatopancreas of *C. maenas*, treated with TN buffer. *In situ* hybridisation using the E2b probe produced no signals within the tissues, indicating absence of P virus. Scale bar = 25 μm.


A
C
B
D

Chapter 5

PRIMARY CULTURE OF HAEMOCYTES FROM MARINE DECAPODS

5.1 INTRODUCTION

Decapod crustaceans possess large populations of haemocytes which circulate in an open vascular system. These cells have a number of functions including blood coagulation, agglutinin formation, and the synthesis, storage and distribution of materials (reviewed by Bauchau, 1981). They are primarily involved in host defence and have phagocytic, encapsulating and lytic activities (Smith & Söderhäll, 1986; Söderhäll & Cerenius, 1992). In most species of crustaceans, haemocytes can be divided into three distinct cell types named hyaline, semi-granular and granular cells respectively (Bauchau, 1981). The hyaline cells are phagocytic and generally characterised by an absence of large granules and prophenoloxidase activity (Söderhäll & Smith, 1983). The semi-granular cells may or may not be phagocytic, depending on species and contain numerous small granules (Bauchau, 1981; Söderhäll & Smith, 1983). They are thought to be the key haemocytes in host defence because of their sensitivity to foreign agents (Smith & Söderhäll, 1986b; Söderhäll & Cerenius, 1992). They release 'communicating signals', by exocytosis upon non-self stimulation, which act on hyaline and/or granular cells (Smith & Söderhäll, 1986; Smith and Chisholm, 1992). Granular cells are generally not phagocytic and possess many large intracellular granules containing prophenoloxidase activating components (Johansson & Söderhäll, 1985; Söderhäll & Smith, 1986). These are released by degranulation on stimulation with proPO proteins (Johansson & Söderhäll, 1985; Söderhäll & Smith, 1986).

Haemocytes in crustaceans are thought to originate from haemopoietic tissue which, in most species, consists of a series of nodules surrounded by a sheath of connective tissue (Ghiretti-Magaldi *et al.*, 1977; Johnson 1980; Bauchau, 1981; Martin *et al.*, 1987). Crustacean

haemocytes were once thought to derive from a single stem cell line generated in haemopoietic tissue (Cuénot, 1891; Kollman, 1908) The haemoblasts (stem cells) then give rise to the hyaline cells which differentiate into semi-granular then granular cells (Bauchau, 1981). This theory, that has been supported by morphological studies of haemopoietic tissue from the shore crab, *C. maenas* (Ghiretti-Magaldi, 1977) and the blue crab, *Callinectes sapidus* (Bodammer, 1978). However, recently, Hose *et al.*, (1990) have proposed an alternative hypothesis, that haemocytes originate from 2 cell lines, one differentiating to form hyaline cells and the other the two types of granular cells. This theory was based on morphological and functional data from the spiny lobster, *Panulirus interuptus*, sheep crab, *Loxorhynchus grandis* and the marine lobster, *Homarus americanus* (Hose *et al.*, 1990). The origin and maturation of haemocytes, however, remains controversial and until experimental studies involving isolated haemopoietic tissues and/or haemocyte cell lines are undertaken the origin and maturation of crustacean haemocytes cannot be firmly established.

There are relatively few reports of invertebrate cell culture methodologies, despite the huge diversity of invertebrate species and their enormous potential as *in vitro* models. As far as crustaceans are concerned, Lu *et al.*, (1995) and Loh *et al.*, (1997) have described primary culture of lymphoid tissue from penaeid shrimps, which was developed for titration of viral pathogens *in vitro*. To date, however, there has been only one previous report of successful primary culture of haemocytes from decapods (Ellender *et al.*, 1992). There are many reasons why crustacean haemocytes are difficult to maintain under prolonged culture conditions. There may be a number of distinct cell populations which have varying physiological requirements *in vitro*. They rarely exhibit

proliferation *in vitro* or *in vivo*, and they are highly sensitive to non-self materials, frequently undergoing degranulation, clotting or cell aggregation upon exposure to trace amounts of bacterial endotoxin (Smith & Söderhäll, 1986).

In this chapter, density gradient centrifugation, an established method for cell separation already applied to crustacean haemocytes (Söderhäll & Smith 1983), was used to isolate the different haemocyte populations of *L. depurator*, determine the yield of separated haemocytes and how the yield varies throughout the year. This is an essential pre-requisite for the development of a cell culture system because crustacean haemocytes are highly labile and capable of spontaneous activation (Söderhäll & Cerenius 1992). The main aim of this chapter is to develop a suitable cell culture system for marine decapods in which high haemocyte viability and functional activity was maintained for a minimum of 14 days. This will have value for a variety of *in vitro* applications, such as the analysis of the non-specific cellular immune processes, the non-sacrificial quantification of viral pathogens, and the evaluation of toxicological effects on immunocompetent cells.

5.2 MATERIALS AND METHODS

5.2.1 Animals

All crabs (*L. depurator* or *C. maenas*) used in experiments were collected from St. Andrews Bay, North Sea each month from 1997 - 1999 and maintained as described in Chapter 2 (section 2.2.1). Only healthy, inter moult crabs, of both sexes were selected, on a monthly basis, for bleeding.

5.2.2 Haemocyte separation

Haemolymph (0.5 ml) was withdrawn into a syringe containing 0.5 ml marine anticoagulant (0.45 M NaCl, 0.1 M glucose, 0.03 M tri-sodium citrate, 0.026 M citric acid, 0.01 M EDTA, pH 4.6; Appendix 1) from the unsclerotised region a main cheliped. Haemocyte populations were separated using a modification of the density gradient separation procedure described by Söderhäll & Smith (1983). The haemocytes of *L. depurator* were separated on preformed gradients of 50% Percoll and those from *C. maenas* on 60 % Percoll (Pharmacia, Uppsala, Sweden) in sterile, filtered 3.2% NaCl (Appendix 4). Each band of haemocytes was carefully removed, following haemocyte separation, placed into sterile 1.5 ml sterile tubes and stored on ice for a maximum of 15 min before use.

5.2.3 Monthly haemocyte counts

Haemocyte counts on separated populations of male or female *L. depurator* haemocytes were performed on a monthly basis to examine variation in the yields of separated haemocytes. To determine the yield of each population of *L. depurator* haemocytes following cell separation, 100 μl of each cell type was diluted in 400 μl of sterile 3.2 % NaCl (1:5 dilution) and counted using an improved Neubauer haemocytometer as described in 2.2.3. Morphology of separated haemocytes was determined using the cytospin method described in 2.2.3.

5.2.4 Culture media preparation

Three commercially available media were selected to test for the culture of crustacean haemocytes, L15 (Sigma, Poole, Dorset, UK), RMPI (Sigma) and MEM (Sigma). The first step in the development of culture

media was to determine the salt supplement required to make each of the test media isosmotic with crab haemolymph. The mean osmolality of crab, *L. depurator*, haemolymph was determined using a Roebling osmometer (Camlab), calibrated with distilled water and a 300 mOsm standard. Each sample was measured twice and the osmolality of the haemolymph was found to be 916 ± 1.8 mOsm kg^{-1}. To determine the salt supplement required to adjust the osmolality of the medium to that of *L. depurator* haemolymph, a range of salt concentrations were prepared to final concentrations of 0.4 M, 1.0 M or 2.0 M NaCl in L15, RPMI or MEM respectively and measured again. The most appropriate NaCl concentration was found to be 0.4M (osmolality = 963 ± 2.3 mOsm kg^{-1}), so for all subsequent experiments each basic medium was supplemented with 0.4M NaCl (final concentration) together with a 1.0 % (final concentration) solution of a penicillin and streptomycin (PS) mix (Sigma).

5.2.5 Hyaline haemocyte culture

Hyaline haemocytes were selected for culture because they readily attach to glass surfaces (Smith & Ratcliffe 1978) and do not contain prophenoloxidase or large granules (Söderhäll & Smith 1983). Accordingly, they do not readily exocytose *in vitro*.

To determine which test medium was most suitable for hyaline haemocyte culture, flasks were set up by adding 0.75 ml of separated *L. depurator* hyaline haemocytes (from males only), suspended in Percoll, to 5 ml of the test medium (either L15, RMPI or MEM containing 0.4 M NaCl and 1 % PS, penicillin-streptomycin mix) in sterile 25 cm^2 flat plastic culture flasks (Corning, Bucks, UK). The flasks were gassed for 5 seconds with 5 % CO_2 - air mix and incubated at 15° C.

L15 containing 0.4 M NaCl and 1% PS was found to be the most suitable of the three media tested (see 5.3.3). To examine the effects of putative growth factors, batches of salt supplemented L15 were prepared with 0, 10 or 20% (final concentration) foetal calf serum (FCS) (Globepharm, University of Surrey, Surrey, UK). To examine the effect of temperature, hyaline cells were cultured in L15 containing 0.4 M NaCl, 1 % PS and 10 % FCS at 5 or 15° C. For all experiments, the medium was changed every 7 days and haemocytes removed at intervals of 2, 4, 7 and 14 days for viability and phagocytosis assays (see below). To remove haemocytes, the medium was aspirated, replaced with 0.5 ml of sterile 0.5 M NaCl, and the haemocytes gently dislodged with a sterile rubber cell scraper (Sigma). A minimum of 5 animals were used for each temperature. Viability was determined at intervals over 14 days by the eosin dye exclusion method (Wilson 1986). For each experiment, approximately 200 haemocytes were counted in duplicate for each crab. A minimum of 5 crabs were used for each treatment. Morphology of cultured haemocytes was determined using the cytospin method described in 2.2.3.

5.2.6 Phagocytosis assay

Since the main role of hyaline haemocytes is phagocytosis (Söderhäll & Smith 1983; Smith and Söderhäll 1983), this characteristic was used to determine functional capability of cultured hyaline haemocytes from *L. depurator* at intervals after 2, 4, 7, 9, 11 or 14 days in culture by evaluation of phagocytic vigour *in vitro*. This was assessed by a modification of the procedure described in Smith & Ratcliffe (1978) and Söderhäll *et al.*, (1986) using the marine bacterium, *Psychrobacter immobilis* (NCIMB 308), as the challenge particle. This organism was

cultured to log phase in marine broth 2216E (Difco, Detroit, Michigan), washed and resuspended to a concentration of 2 x 10^6 ml^{-1} in sterile, filtered 3.2 % NaCl as described in Chisholm & Smith (1991). Monolayers of the haemocytes were prepared by culturing haemocytes for 2, 4, 7, 9, 11 or 14 days at 5 or 15° C. At each time point, the cells were removed from the flasks as described above, and 200 μl of each suspension was pipetted onto clean, pyrogen free coverslips. The cells were allowed to attach to the glass surface for 20 min at 20° C and were washed twice with sterile 0.5 M NaCl before being overlaid with 100 μl of the prepared bacterial suspension. The cell-bacteria mixtures were incubated in a moist chamber for 3 h at 20° C. They were then washed thoroughly with sterile 0.5 M NaCl to remove unattached bacteria and finally fixed for 20 min in 10 % formaldehyde in sea water. The monolayers were scrutinised under phase contrast optics and the number of cells containing one or more intracellular bacteria, assessed using the criteria given in Smith & Ratcliffe (1978) was determined from a minimum of 100 haemocytes per coverslip. Duplicate coverslips were counted for each time period.

5.2.7 Semi-granular cell culture

Having established optimal conditions for hyaline haemocyte viability and functionality in primary culture, an additional experiment was undertaken to ascertain if semi-granular cells could be successfully cultured under the same conditions. Semi-granular haemocytes from *L. depurator* were removed from Percoll gradients following separation (described in 5.2.2) and maintained in L 15 containing 0.4 M NaCl, 1 % PS and 10 % FCS at 15° C for 2, 7 and 14 days as described in 5.2.3. Viability and morphology of cultured semi-granular haemocytes was determined at these intervals as described for hyaline haemocytes in 5.2.5. Granular

haemocytes were not cultured because they do not readily attach to surfaces making them less amenable for culture.

5.2.8 Species comparison

To ascertain whether the culture method ultimately derived for *L. depurator* hyaline and semi-granular haemocytes had broad applicability to other marine brachyurans, additional cultures were set up with hyaline cells from the shore crab, *C. maenas*. Separated hyaline haemocytes were obtained as described in Söderhäll & Smith (1983a) and Söderhäll *et al.*, (1986), using 60 % Percoll gradients (Appendix 4) for haemocyte separation, and maintained in sterile L15 medium supplemented with 0.4 M NaCl, 1 % PS and 10 % FCS, again over 14 days at 15° C. Cells were harvested after 2, 7 or 14 days and viability assessed as described in 5.2.5.

5.2.9 Autoradiography

To assess the possibility of developing long-term haemocyte cultures, populations of separated *L. depurator* haemocytes were examined for evidence of haemocyte proliferation. Haemolymph was extracted from 5 healthy, male, inter moult crabs, previously quarantined for two weeks and separated on 50 % Percoll gradients as described in 5.2.2. One millilitre of each cell type was added to a sterile tissue culture flask containing 2.5 ml of L 15 supplemented with 0.4 M NaCl, 10 % FCS and 1 % PS. Tritiated thymidine (^{3}H tdr) was added to each flask to a final concentration of 0.3 kbeq ml-1 (i.e. 350 μl of 300 kbeq ml-1 stock) and cells were incubated at 5° C for 24 hours. Any haemocytes that had attached during the incubation period were gently scraped into the medium and

500 μl from each flask were centrifuged onto glass slides as described in 2.2.3, air-dried and fixed in methanol.

In a darkroom, emulsion (Ilford K2 emulsion in gel form) was melted to a smooth consistency in a sterile measuring cylinder at 45° C. One volume of distilled water containing 1 % glycerol was added to the cylinder and this solution placed in a glass beaker at 45° C. Slides were dipped in the emulsion and the side without cells wiped clean. The slides were left to dry in the dark, for 4 hours at 20° C before being placed in racks, sealed to exclude light and incubated for 4 days at 5° C. Developer was prepared by adding 160 g of Kodak D19 developer to 1 litre of distilled water at 38° C in the dark. Fixative was prepared by diluting Kodak Unifix 1:4 in distilled water (in the dark). Slides were placed in developer for 3.5 min and rinsed thoroughly in tap water before being placed in fixative for 10 min and thoroughly rinsed again.

In the light, slides were allowed to dry before staining by the Romanovsky method and mounting in DePeX (BDH). The total number of cells from five fields of view per slide were counted and the numbers of radio-labelled cells noted to determine percentage proliferation of each cell type.

5.2.10 Statistics

Haemocyte counts are expressed as mean cells $ml^{-1} \pm$ standard error of the mean. All viability data are expressed as mean percentage viability ± standard error of the mean. One way ANOVA was performed on square root transformed monthly haemocyte data (Sokal & Rohlf 1981). Comparisons of haemocyte viability in various media and at different supplement levels were performed using ANOVA on arcsine transformed data (Sokal & Rohlf 1981). The effect of temperature on

haemocyte viability and comparisons of haemocyte viability between species were analysed using Student's t-tests on paired or unpaired, arcsine transformed data where appropriate (Sokal & Rohlf 1981). Phagocytosis data are expressed as mean percentage phagocytosis ± standard error of the mean. Phagocytic uptake of bacteria by cultured haemocytes was compared to uptake in freshly extracted haemocytes using Student's t-tests on unpaired, arcsine transformed data (Sokal & Rohlf 1981).

5.3 RESULTS

5.3.1 Haemocyte separation

The three types of circulating haemocytes, from *L. depurator*, were separated on 50% Percoll gradients (Fig 5.1). Hyaline haemocytes formed the uppermost band, semi-granular the middle band and granular haemocytes the lowest band (Fig 5.1). The three haemocyte populations from *L. depurator* had different morphological characteristics. The hyaline haemocytes were characterised by a large nucleus and slight orange appearance in the cytoplasm (Fig 5.2A). The semi-granular haemocytes were characterised by a smaller nucleus than hyaline haemocytes and slight granular appearance in the cytoplasm (Fig 5.2B). The granular haemocytes had a small, compact nucleus and very granular cytoplasm (Fig 5.2C).

When the yield of haemocytes following separation were compared, no significant difference was observed between haemocyte populations extracted from male or female *L. depurator*. In separated hyaline haemocyte populations, the average haemocyte number was 1.72

$\pm$ 0.28 x 10^6 ml^{-1} for males and 1.58 $\pm$ 0.26 x 10^6 ml^{-1} for females (Fig 5.3). Separated semi-granular haemocytes were the most abundant haemocyte type with an average of 2.98 $\pm$ 0.31 x 10^6 haemocytes ml^{-1} for male crabs and 2.74 $\pm$ 0.30 haemocytes x 10^6 ml^{-1} for female crabs (Fig 5.3). Separated granular haemocytes were the least abundant haemocytes, mean haemocyte number was 1.09 $\pm$ 0.15 x 10^6 ml^{-1} for males and 1.24 $\pm$ 0.24 x 10^6 haemocytes ml^{-1} for females (Fig 5.3).

5.3.2 Monthly haemocyte counts

The yield of each haemocyte population, following separation on Percoll gradients showed significant monthly variations ($P < 0.05$) (Fig 5.4). The lowest separated hyaline haemocyte numbers were observed in June, where the average haemocyte number was 0.48 $\pm$ 0.14 x 10^6 ml^{-1} and highest hyaline haemocyte numbers were in October, 3.49 $\pm$ 1.82 x 10^6 ml^{-1} ($P < 0.05$) (Fig 5.4A). The lowest numbers of semi-granular haemocyte numbers also occurred in June, where the average haemocyte number was 1.59 $\pm$ 0.42 x 10^6 ml^{-1}, and highest were in December, 5.93 $\pm$ 1.47 x 10^6 ml^{-1} ($P < 0.05$) (Fig 5.4B). The lowest numbers of granular haemocytes were observed August, 0.39 $\pm$ 0.08 x 10^6 ml^{-1}, and highest were in November, 1.10 $\pm$ 0.36 x 10^6 ml^{-1} ($P < 0.05$) (Fig 5.4C).

5.3.3 Hyaline haemocyte culture

The hyaline haemocytes from *L. depurator* survived, but did not grow, in each of the three culture media tested (Fig. 5.5). After 2 days, haemocyte viability was 70.1 $\pm$ 5.8 % in L15, 51.9 $\pm$ 3.6 % in RPMI and 49.3 $\pm$ 12.4 % in MEM (Fig 5.5). However, over the next three days viability of the haemocytes in RPMI or MEM fell dramatically to 16.7 $\pm$ 4.7 % and 19.2 $\pm$ 4.2 % respectively. This was significantly lower than haemocyte

viability in L15 medium (72.7 ± 5.2 %) over the same time (5 days) ($P < 0.05$). By 7 days, mean haemocyte viability was 63.1 ± 7.94 % in L15 but only 9.9 ± 4.19 % in RPMI and 0.0% in MEM (Fig 5.5). Since good haemocyte viability was obtained in L15 media, it was selected for use in further experiments.

Inclusion of FCS in the salt amended L15 medium significantly promoted viability of the cultured hyaline haemocytes. After 2 days, haemocyte viability increased from 68.2 ± 4.7 % in unsupplemented media to 88.8 ± 2.3 % with a 10% FCS supplement and 88.9 ± 2.2 % with a 20 % FCS supplement (Fig 5.6). Importantly, inclusion of FCS prolonged haemocyte survival over the following 12 days so that by day 14, overall viability was 39.1 ± 11.3 % without FCS, but 72.0 ± 8.2 % in 10 % FCS ($P < 0.05$) and 78.6 ± 5.2 % in 20 % FCS ($P < 0.05$) (Fig. 5.6). There was no significant difference in haemocyte viability between 10 and 20 % FCS, but as haemocyte clumping sometimes occurred when a supplement of 20 % FCS was used, all subsequent experiments used only 10 % FCS.

Experiments to investigate the effect of incubation temperature on survival of *L. depurator* hyaline haemocytes *in vitro* revealed that haemocyte viability remained high after 14 days in culture at both temperatures tested. Haemocytes were maintained in L15 containing 0.4 M NaCl, 1 % PS and 10 % FCS and at 2 days, viability was 92.9 ± 1.4 % at 5° C and 88.8 ± 2.2% at 15° C; at 7 days it was 83. 9 ± 2.2% at 5° C and 83.5 ± 2.0% at 15° C; and at 14 days was 71.8 ± 8.5% at 5° C and 72.0 ± 8.2% at 15° C (Fig 5.7). There was no significant difference between the values obtained for the two temperatures at each time point.

In L15 media supplemented with 0.4 M NaCl, 1 % PS and 10 % FCS at 15° C, haemocytes attached to the base of flasks to form monolayers (Fig 5.8A). Cytospin preparations of the cultured haemocytes showed that

the cultured haemocytes were intact, with no sign of necrosis, pycnosis or contamination (Fig 5.8B).

5.3.4 Phagocytosis assay.

Hyaline haemocytes from *L. depurator* have the ability to phagocytose the marine bacterium, *P. immobilis in vitro*. The mean percentage uptake of this bacterium in freshly extracted hyaline haemocytes was 18.8 ± 4.9%. Cultured hyaline haemocytes from *L. depurator* were found to retain their ability to phagocytose *P. immobilis*, over the full 14 day culture period (Fig. 5.9). Haemocytes cultured for two days showed considerably elevated levels of uptake, with 35.7 ± 5.5 % of those haemocytes previously maintained at 5° C and 27.9 ± 2.8 % of those haemocytes cultured at 15° C found to contain one or more bacteria (Fig 5.9), both values are significantly higher than the levels of ingestion observed for freshly extracted phagocytes ($P < 0.01$), but not significantly different from each other. Phagocytic rates declined to around 25 % with the 4, 7, or 9 day old haemocytes, although the 15° C incubated haemocytes tended to show similar or slightly higher levels of uptake than the 5° C haemocytes (Fig 5.9). Phagocytic rates remained close to ca 17 % with the 11 and 14 day haemocytes, with the mean uptake by 14 day haemocytes found to be 18.2 ± 5.5% at 5° C and 16.6 ± 3.0% at 15° C (Fig 5.9). Values from day four to day fourteen were not significantly different to each other or to the level of uptake recorded for freshly extracted haemocytes.

5.3.5 Semi-granular haemocyte culture

Having established good viability of *L. depurator* hyaline haemocytes in L15 medium containing 0.4 M NaCl, 1 % PS and 10 % FCS, this medium was used to evaluate survival of separated semi-granular haemocytes from *L. depurator* at 15° C. Eosin Y dye exclusion measurements showed that viability of *L. depurator* semi-granular haemocytes was 79.5 ± 3.49 % after 2 days, 77.1 ± 8.30 % after 7 days and after 87.2 ± 4.05 % after 14 days (Fig. 5.10). There was no significant difference in haemocyte viability over the culture period.

5.3.6 Species comparison

Having established good viability of *L. depurator* hyaline and semi-granular haemocytes in L15 medium containing 0.4 M NaCl, 1 % PS and 10% FCS, this medium was used to evaluate survival of separated hyaline haemocytes from *C. maenas* at 15° C. Eosin Y exclusion measurements showed that viability of the *C. maenas* hyaline haemocytes was 84.3 ± 4.3% after 2 days in culture, 88.1 ± 10.3% after 7 days and 84.2 ± 5.25% after 14 days (Fig 5.11). These values compare favourably with survival rates for *L. depurator* hyaline and semi-granular haemocytes (above). The hyaline haemocytes from *C. maenas,* also formed monolayers on the base of culture flasks (Fig 5.12A). The appearance of the monolayer was similar to that formed by *L. depurator* hyaline haemocytes, although *C. maenas* hyaline haemocytes tended to spread more than those from *L. depurator* (Fig 5.12A). Cytospin preparations of the cultured haemocytes showed that haemocytes were intact, with no sign of necrosis, pycnosis or contamination (Fig 5.12B).

5.3.7 Autoradiography

Positive uptake of ^{3}H, indicative of DNA synthesis and proliferation, was observed in semi-granular haemocytes of all five *L. depurator* examined and was characterised by silver precipitate on the nucleus of actively proliferating haemocytes (Fig 5.13A). ^{3}H uptake was also observed in the granular haemocytes of one *L. depurator* (Fig 5.13B) although it was not observed in the hyaline haemocytes from any of the other *L. depurator*. The percentage of proliferating haemocytes was low, approximately 1 % of semi-granular haemocytes in each *L. depurator* and 1 % in the granular haemocytes from one *L. depurator* (Table 5.1).

5.4 DISCUSSION

The swimming crab, *L. depurator* has three populations of circulating haemocytes which can be separated on 50 % Percoll gradients by the density centrifugation method. The separation of haemocyte populations is an essential prerequisite for experimental investigations, particularly haemocyte culture development, because in mixed populations they are prone to clotting (Söderhäll & Smith, 1986; Söderhäll & Cerenius, 1992). Analysis of the yields of haemocytes following separation revealed that semi-granular haemocytes were the most abundant type followed by hyaline haemocytes with granular haemocytes usually least abundant. Interestingly, when male and female separated haemocyte populations were examined, no significant difference was found in the yield of haemocytes. This has important implications when considering the set up of haemocyte cultures, clearly haemolymph from males and females can produce good yields of haemocytes, however, only haemocytes from males were used in the

subsequent development of the haemocyte culture system to eliminate possible variation due to differences in reproductive effort.

Interestingly, the yield of each haemocyte type following separation on Percoll gradients showed monthly variations. The lowest yields occurred in the summer and highest in the winter. There are many possible factors that could influence haemocyte number including life history, (Young & Pearce, 1975), moult stage (Hose *et al.*, 1992), environmental quality (Smith & Johnson, 1992), temperature (Truscott & White, 1990), handling stress (Stewart *et al.*, 1966; Hamann, 1975) and antigenic challenge (Cornick & Stewart, 1968; Sawyer *et al.*, 1970; Smith & Ratcliffe, 1980; Persson *et al.*, 1987; Lorenzon *et al.*, 1999). However, to fully understand factors affecting haemocyte number and seasonal variations, total and differential haemocyte counts are required. Since seasonal variations observed in this study were on separated haemocytes extracted from Percoll gradients, no direct inferences can be made as to the composition of haemocytes in the haemolymph of animals. However, understanding variation in yields of haemocytes following haemocyte separation provides important data, indicating times of the year when difficulties may be encountered setting up haemocyte cultures, or other *in vitro* experiments, due to low haemocyte yields. This information has proved essential for the development of a primary haemocyte culture system for crustaceans.

This chapter describes a method for the *in vitro* culture of the hyaline and semi-granular haemocytes from *L. depurator* and hyaline haemocytes from *C. maenas* for a medium term period of 14 days. This method is based on L15 medium supplemented with NaCl, antibiotics and 10 % FCS. Crucially, this medium maintains high haemocyte viability for both species over the full incubation period, and the

haemocytes retain their ability to phagocytose bacteria *in vitro*. This is one of the first reports of a primary culture system for haemocytes of decapod crustaceans which favours both survival and functional activity of circulating blood haemocytes for an extended time.

L15, the most suitable medium for the culture of the haemocytes in this study, has also been used successfully for the culture of shrimp nerve, lymphoid or ovary tissues (Nadala *et al.*, 1993). By contrast, RPMI 1640 medium has been preferred for culture of ascidian lymphoid haemocytes (Raftos *et al.*, 1990; Rinkevitch & Rabinowitz, 1993; Smith & Peddie, 1995; Peddie *et al.*, 1995). The culture system used in the present study proved effective in keeping growth of contaminant micro-organisms in check. Other workers, notably, Ellender *et al.*, (1992) and Pomponi *et al.*, (1997) have experienced serious problems of high levels of contamination in some of their cultures. Not only is it essential to avoid contamination in haemocyte or tissue cultures over prolonged periods to ensure that the haemocytes of interest are not overgrown, but also, for immunological analysis, it is vital to ensure that the haemocytes environment remains as near endotoxin-free as possible. In the present study, as the haemocytes did not loose their ability to phagocytose bacteria *in vitro*, it is likely that they had not been 'spontaneously' activated by non-self materials during the culture period.

The present study also differs from earlier reports in incubating the haemocyte cultures at 5 and 15° C, temperatures much lower than the optimal temperatures (25-32° C) found for shrimp haemocytes by Ellender *et al.* (1992). These temperatures (5 and 15° C) were selected as experimental temperatures in the present study because *L. depurator* is a thermo-conforming invertebrate, which on account of its shallow water epibenthic habit, routinely encounters environmental temperatures

ranging from 3° C to 18° C (Hayward, 1990). For specimens living in the eastern North Sea, typical winter temperatures are around 5° C while summer temperatures in inshore waters average ca 15° C (Hayward, 1990). Thus 5 and 15° C are the normal seasonal temperatures encountered by *L. depurator*. In the present study, no significant difference was observed between the viability of the haemocytes incubated at 5° C and 15° C, showing that the hyaline haemocytes of *L. depurator* have considerable plasticity in temperature tolerance *in vitro*. Such plasticity has been observed with the cells from other cold water species, for example, salmonid haemocytes will grow well at 20° C, tolerate 4° C but die above 27° C (Wolf 1979). This indicates that the optimal incubation temperature for haemocyte culture of poikilothermic animals varies from species to species in accordance with the normal environmental temperature of the host.

L. depurator hyaline haemocytes showed good survival and phagocytic responsiveness following incubation at 5 or 15° C. Most previous physiological *in vitro* studies of temperate water marine crustacean haemocytes have been carried out at 15 or 20° C (Smith & Söderhäll, 1983; Chisholm & Smith, 1992, Bell & Smith 1993) and, at this temperature, uptake of bacteria has been found to be ca 20%; a value close to that obtained with freshly collected haemocytes from *L. depurator* in the present investigation. No attempt was made to measure uptake of bacteria by cultured *L. depurator* semi-granular haemocytes or hyaline haemocytes from *C. maenas* in this study, because they were used only to assess the broad applicability of the medium. Interestingly, phagocytosis by 2 day cultured *L. depurator* haemocytes was found to be higher than freshly collected haemocytes. One explanation for this phenomenon is that there was some pre-selection of phagocytic haemocytes during the

first 48 h *in vitro*, producing apparently greater rates of uptake than uncultured haemocytes. Certainly, there was some loss of adherent haemocytes over the 14 day culture period so it is possible that the weakly adherent haemocytes are not phagocytically capable; a hypothesis consistent with the suggestion of Thornqvist *et al.* (1994) that adhesion molecules function as opsonins in crustaceans.

In this chapter, it has been shown that approximately 1 % of circulating semi-granular haemocytes are in a proliferative state in the 24 h following withdrawal of haemolymph from the animal. This level of haemocyte proliferation is very similar to the 1-2 % reported from the circulating haemocytes of penaeid shrimps (Ellender *et al.*, 1992; Sequiera, *et al.*, 1996). The discovery of circulatory haemocytes in a proliferate state indicates that it may be possible to stimulate haemocytes to divide *in vitro*, an essential pre-requisite for the establishment of a crustacean haemocyte haemocyte line. Mitogens including concanavalin A, phytohaemagglutinin and lipopolysaccharide have been used to stimulate cell mitosis in blood cells from the ascidian, *Ciona intestinalis* (Peddie *et al.*, 1995), so it may be possible to stimulate crustacean haemocytes to divide in culture by use of such mitogens.

To conclude, the work described in the present chapter goes a long way to address the problem of the lack of medium term primary culture technologies for the haemocytes of decapod marine invertebrate animals. The above system is simple to set up, inexpensive to maintain and requires minimal attention once established. It can be used to investigate several aspects of immunity, pathology and ecotoxicology in marine crustaceans. Such information is particularly important to the growing aquaculture industry, where there is a distinct need for methods of

disease diagnosis, disease control and non-sacrificial evaluation of the effect of environmental quality on health.

Figure 5.1 Separation of haemocytes from male *L. depurator* on a 50 % Percoll gradient. Haemocytes separate into three distinct bands. A band of flocculated material forms the uppermost band; Hyaline haemocytes form the first band of haemocytes; semi-granular haemocytes form the middle band of haemocytess and granular haemocytes form the lowest band of haemocytes.
The separation was performed on a pre-formed density gradient consisting of 50 % Percoll stock (1 part 32.0 % sterile NaCl : 9 parts Percoll) and 50 % of sterile 3.2 % NaCl.

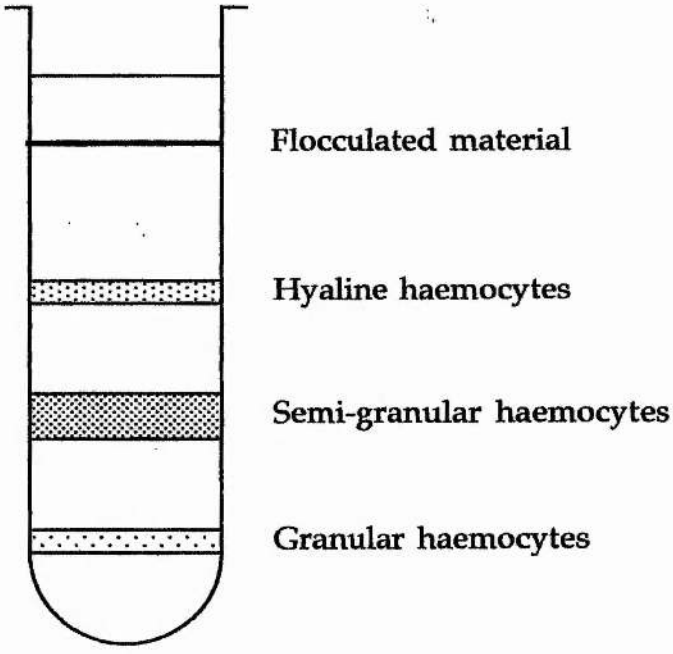
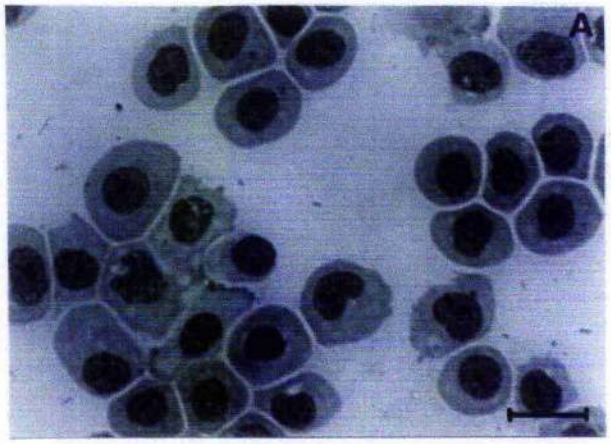

Flocculated material
Hyaline haemocytes
Semi-granular haemocytes
Granular haemocytes

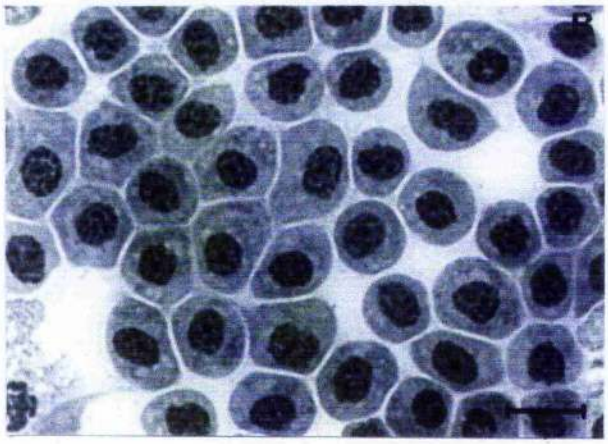
Figure 5.2 Haemocyte morphology of separated haemocyte populations from male *L. depurator*.

A) Cytospin preparation of hyaline haemocytes separated on 50 % Percoll gradient.
Hyaline haemocytes have large nuclei and a small amount of cytoplasm which stains blue with Romanovsky stain, indicating high levels of mRNA. Scale bar = 5.0 μm.

B) Cytospin preparation of semi-granular haemocytes separated on 50 % Percoll gradient.
Semi-granular haemocytes also have relatively large nuclei and have small granules which give the cells a slightly orange appearance with Romanovsky stain. Scale bar = 5.0 μm.

C) Cytospin preparation of granular haemocytes separated on 50 % Percoll gradient.
Granular haemocytes have relatively small compact nuclei and dense cytoplasm containing large granules which stain intensely orange with Romanovsky stain. Scale bar = 5.0 μm.


A


B

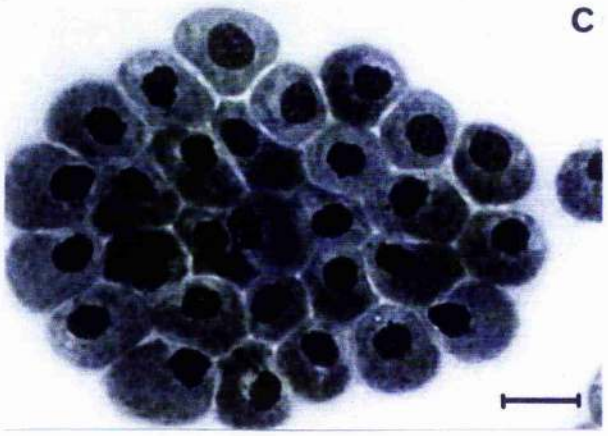

C

Figure 5.3 Abundance of the three haemocyte populations found in *L. depurator* from (■) males and (▨) females. H = hyaline haemocytes, S = semi-granular haemocytes and G = granular haemocytes.

Abundance is expressed as mean haemocytes ml^{-1} ± standard error of the mean. n = 129.

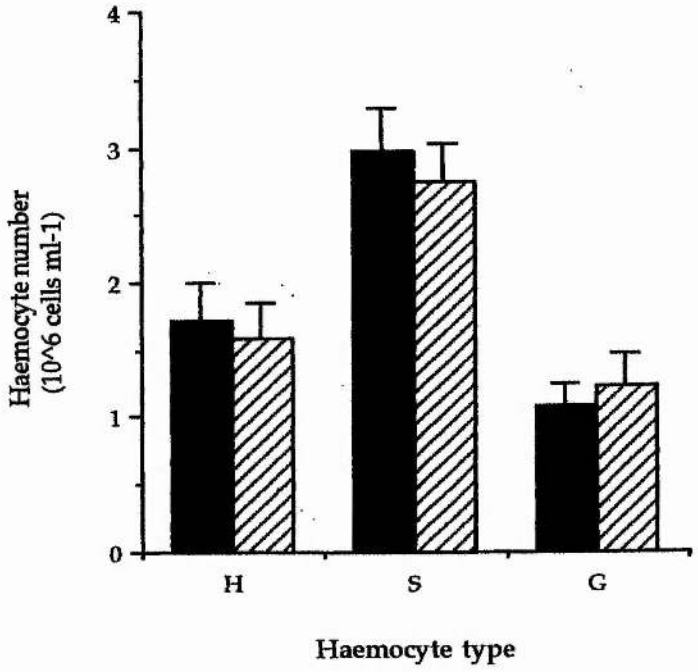

Haemocyte number
(10^6 cells ml-1)
4
3
2
1
0
H
S
G
Haemocyte type

Figure 5.4 Variation in numbers of separated haemocytes, from male *L. depurator,* throughout the year.

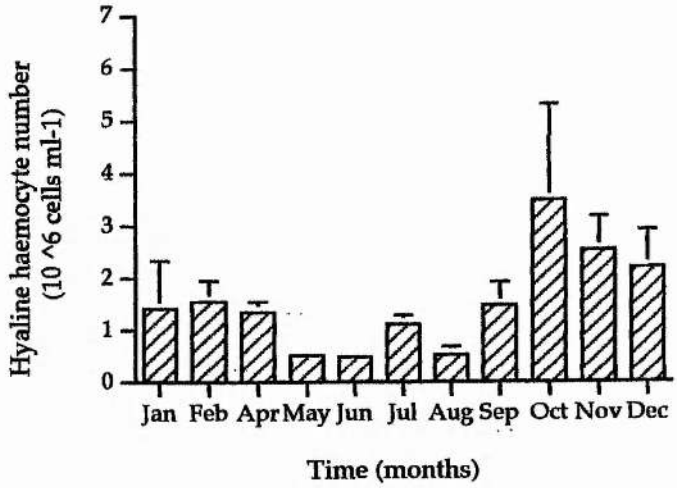
A. Hyaline haemocyte number (▨) from January - December (excluding March due to bad weather). n = 129.

B. Semi-granular haemocyte numbers (▧) from January - December (excluding March due to bad weather). n = 129.

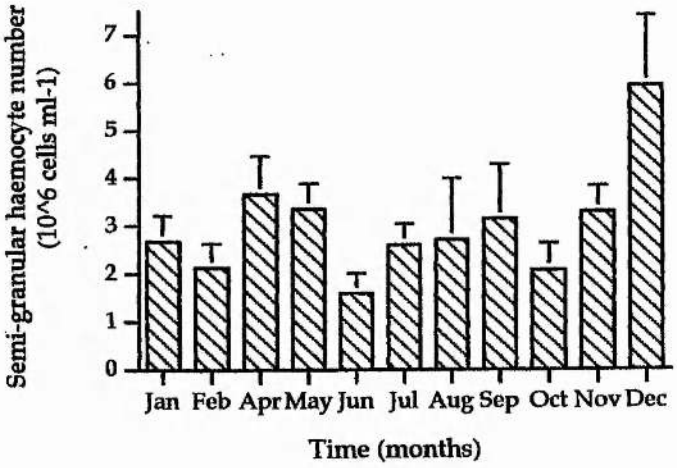
C. Granular haemocyte numbers (■) from January - December (excluding March due to bad weather). n = 129.

All values are expressed as haemocytes $ml^{-1} \times 10^6$ and are means ± standard error of the mean.

A. Hyaline haemocytes



B. Semi-granular haemocytes



C. Granular haemocytes

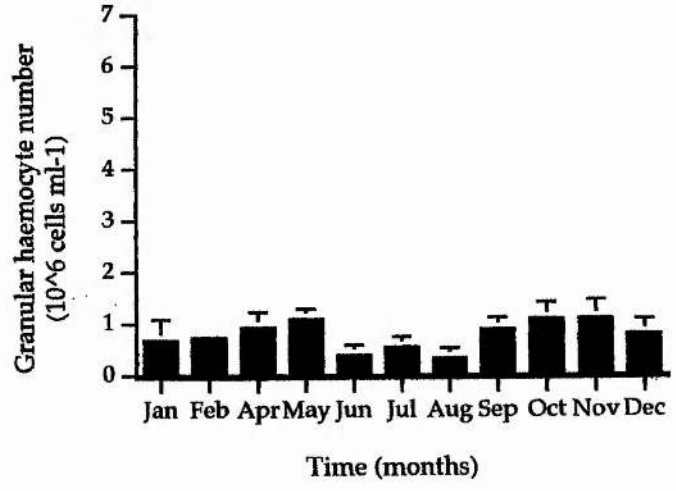


Figure 5.5 Viability of hyaline haemocytes from male *L. depurator* in different media *in vitro*. L15 (-■-), RPMI 1640 (-◆-) or MEM (-●-). All were supplemented with sterile 0.4 M NaCl and 1 % PS (antibiotics) (final concentrations). Values given are means ± SE (n = 5).

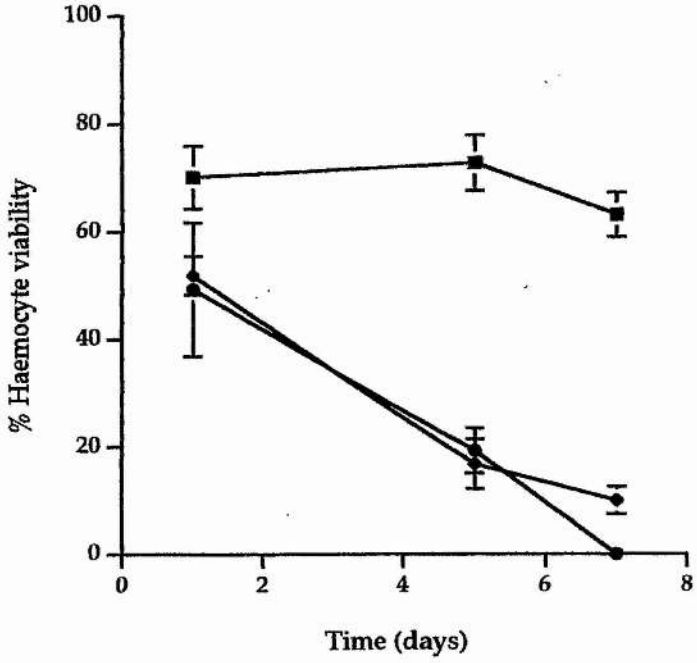

100
80
60
40
20
0
% Haemocyte viability
0
2
4
6
8
Time (days)

Figure 5.6 Viability of cultured hyaline haemocytes from male *L. depurator* in 0 (-■-), 10 (-◆-) or 20% (-●-) FCS. The culture media was L15 medium supplemented with sterile 0.4 M NaCl and 1 % PS (antibiotics) (final concentrations). Values are means ± SE (n = 5).

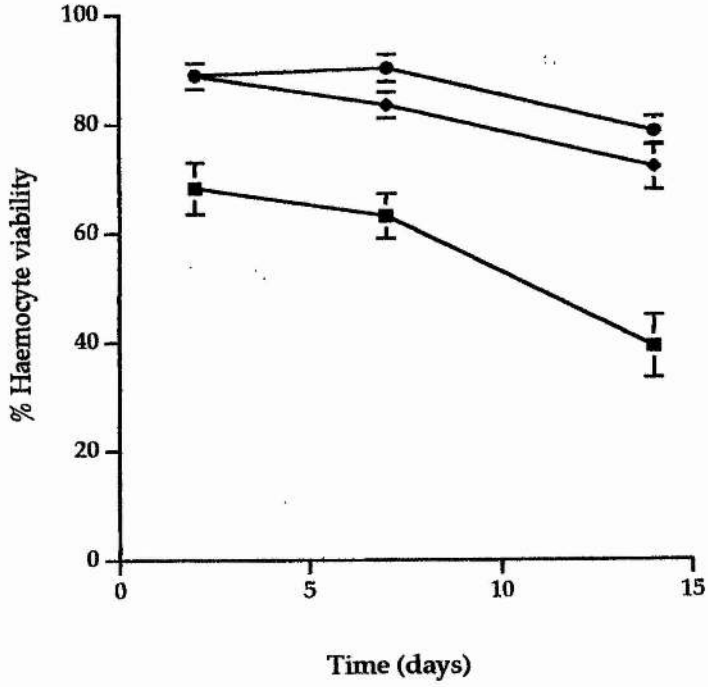

100
80
60
40
20
0
% Haemocyte viability
0
5
10
15
Time (days)

Figure 5.7 Viability of cultured hyaline haemocytes from male *L. depurator* at different temperatures. The haemocytes were cultured at 5 (■) or 15°C (▨) in L15 medium containing sterile 0.4 M NaCl, 10 % FCS and 1% PS (antibiotics) (final concentrations). Values are means ± SE (n = 5).

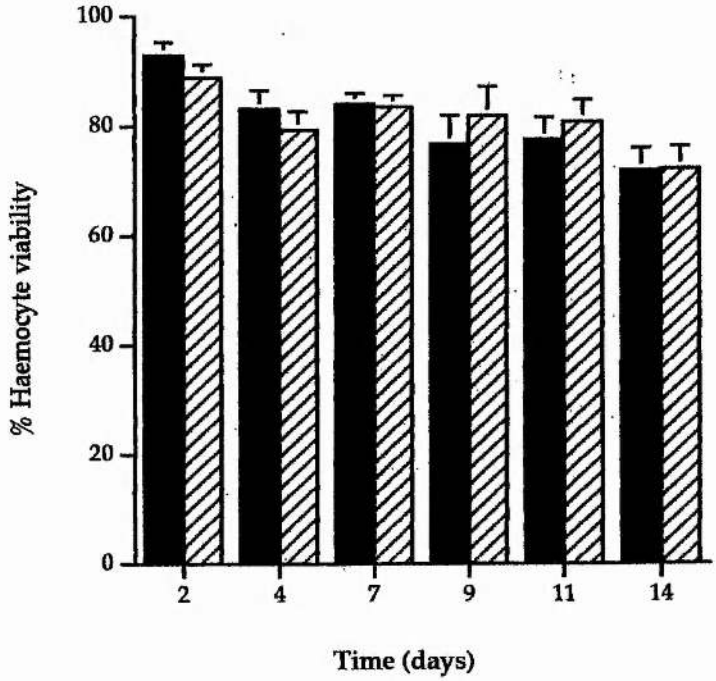
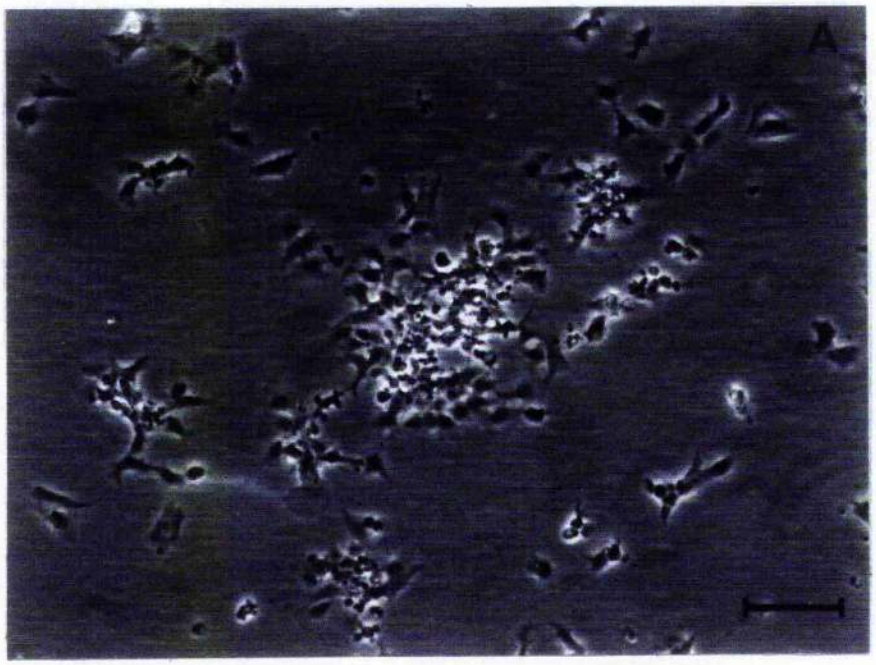

100
80
60
40
20
0
% Haemocyte viability
2
4
7
9
11
14
Time (days)

Figure 5.8 Appearance of cultured hyaline haemocytes from male *L. depurator in vitro.*

A . Hyaline haemocytes after 2 days *in vitro.* Phase contrast optics. Scale bar = 20 μm.

B. Cytospin preparation of hyaline haemocytes after 7 days *in vitro* Romanovsky stain. Scale bar = 10 μm.


A

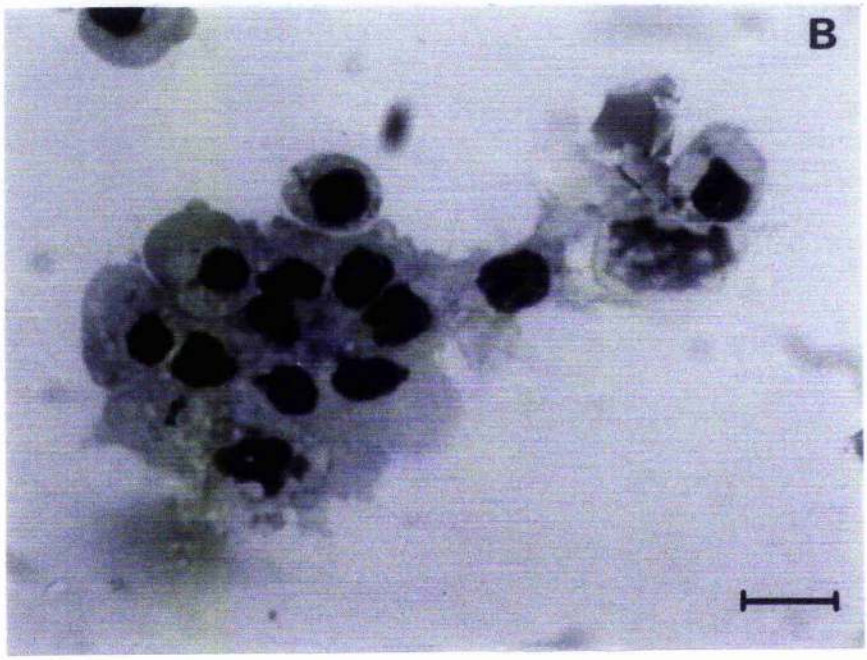

B

Figure 5.9 Phagocytic uptake of the marine bacterium, *Psychrobacter immobilis in vitro* by cultured hyaline haemocytes from male *L. depurator* at 5 (■) or 15°C (▨). The haemocytes were cultured in L15 medium containing sterile 0.4 M NaCl, 10 % FCS and 1% PS (antibiotics) (final concentrations). Values are means ± SE (n = 5).

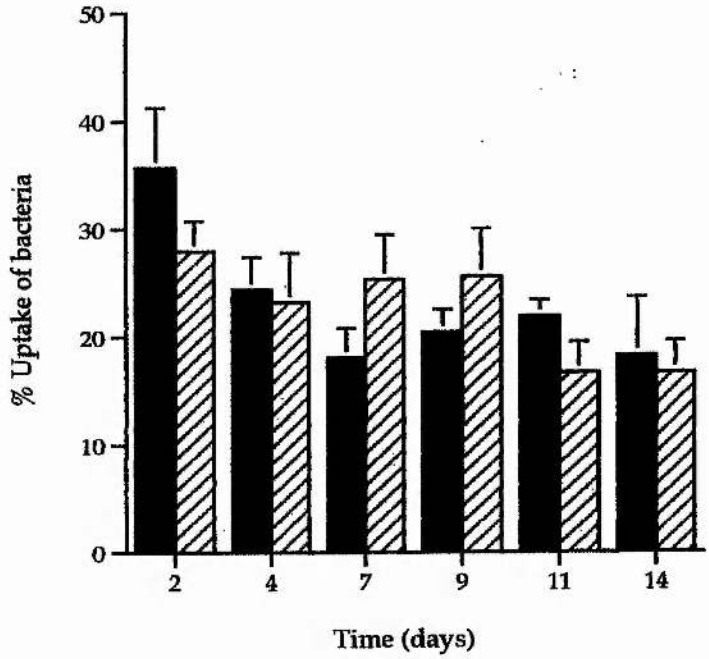

50
40
30
20
10
0
% Uptake of bacteria
2
4
7
9
11
14
Time (days)

Figure 5.10 Viability of cultured semi-granular haemocytes from male *L. depurator in vitro*. The culture media was L15 medium supplemented with sterile 0.4 M NaCl, 10 % FCS and 1% PS (antibiotics) (final concentrations). Values are means ± SE (n = 5).

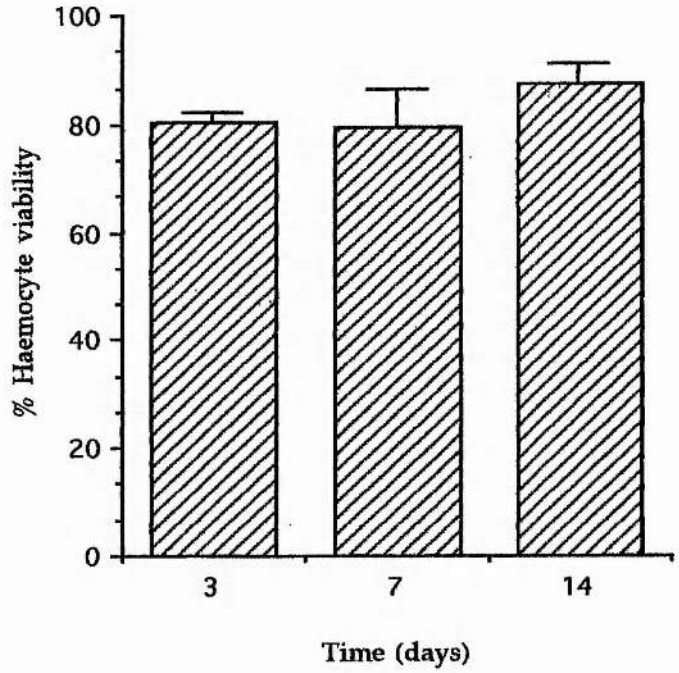

100
80
60
40
20
0
% Haemocyte viability
3
7
14
Time (days)

Figure 5.11 Viability of cultured hyaline haemocytes from male *C. maenas in vitro*. The culture media was L15 medium supplemented with sterile 0.4 M NaCl, 10 % FCS and 1 % PS (antibiotics) (final concentrations). Values are means ± SE (n = 5).

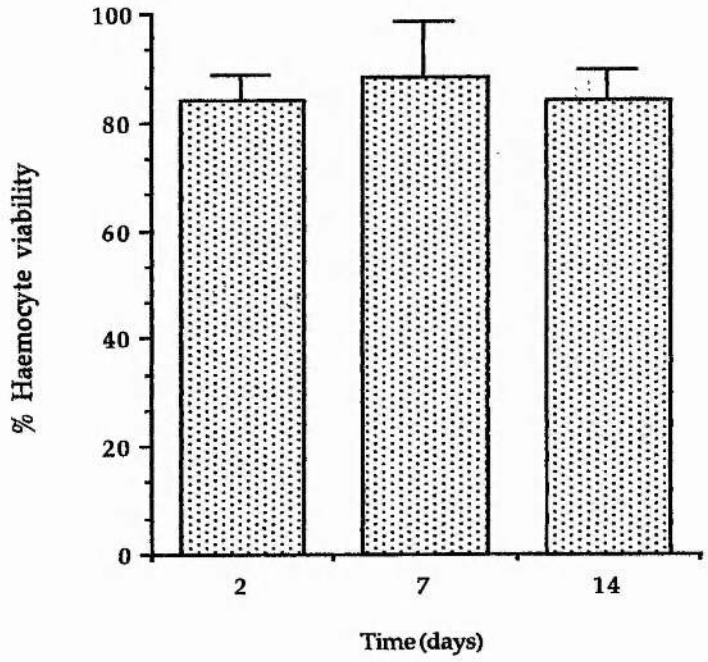
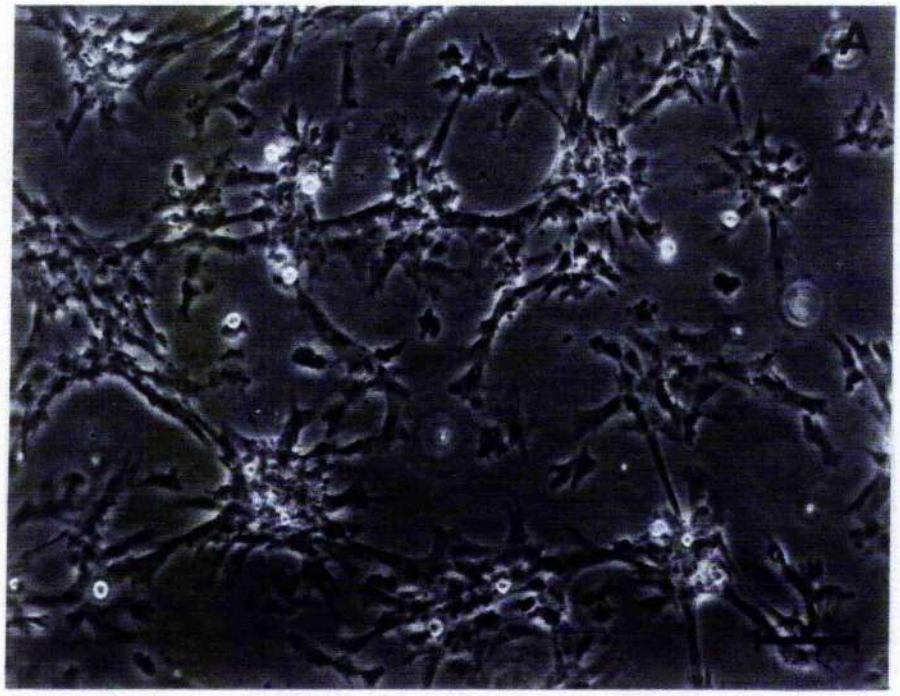

100
80
60
40
20
0
% Haemocyte viability
2
7
14
Time (days)

Figure 5.12 Appearance of cultured hyaline haemocytes from male *C. maenas in vitro.*

A Hyaline haemocytes from *C. maenas* after 2 days *in vitro.* Phase contrast optics. Scale bar = 20 μm.

B. Cytospin preparation of hyaline haemocytes from *C. maenas* after 7 days *in vitro* Romanovsky stain. Scale bar = 10 μm.


A

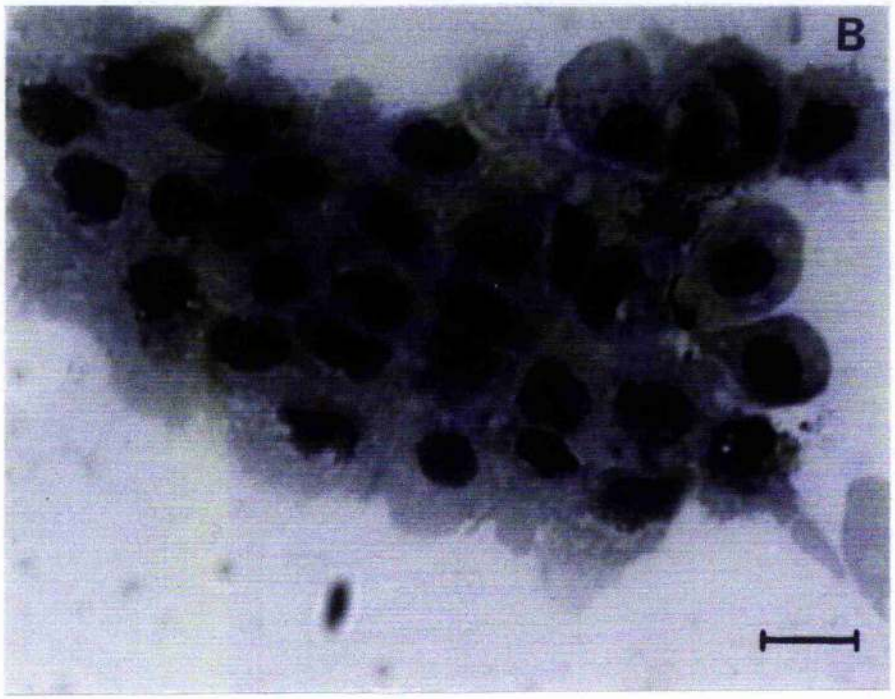
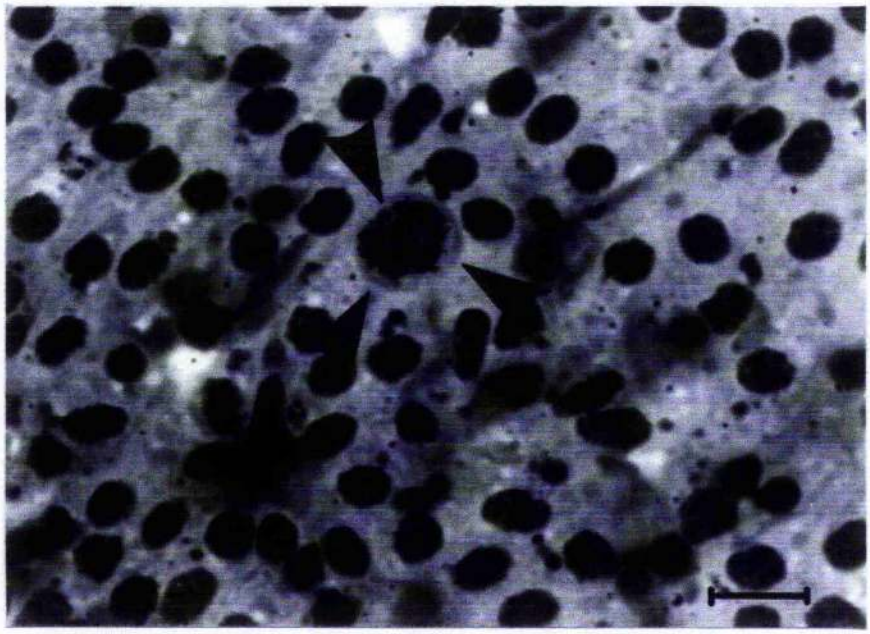

B

Figure 5.13 Autoradiographs of semi-granular haemocytes (A) and granular haemocytes (B). Proliferating cells are characterised by silver, granular precipitate over dividing nuclei (arrows). Scale bar = 5.0 μm.

A



B

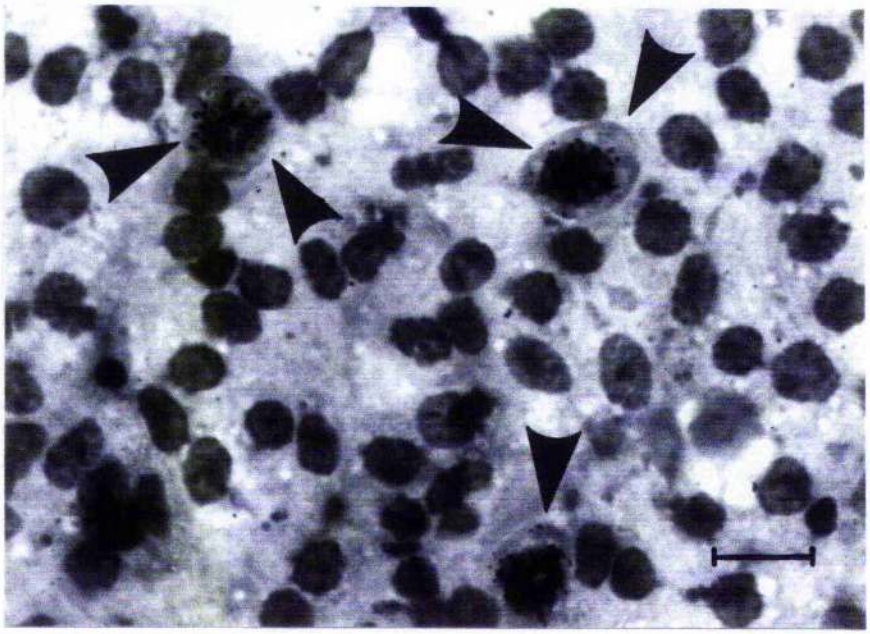


Table 5.1 Haemocyte proliferation in hyaline, semi-granular and granular cells from healthy, male, *L. depurator*; maintained for two weeks. Percentage proliferation is expressed as % of proliferating haemocytes observed from five fields of view for each animal.

Table 5.1

HAEMOCYTE TYPE	ANIMAL	% PROLIFERATION	TOTAL HAEMOCYTES
Hyaline	1	0.0	220
	2	0.0	406
	3	0.0	371
	4	0.0	637
	5	0.0	424
Semi-granular	1	1.0	298
	2	2.0	164
	3	1.0	209
	4	0.0	184
	5	1.0	197
Granular	1	1.0	850
	2	0.0	326
	3	0.0	181
	4	0.0	155
	5	0.0	133

Chapter 6

PRELIMINARY INVESTIGATIONS ON THE PROPAGATION OF P VIRUS *IN VITRO*

6.1 INTRODUCTION

The development of suitable cell culture technologies, or established cell lines of crustacean tissues is essential if progress is to be made in the treatment and control of viral infections in Crustacea. Cell culture technology has a wide range of applications in virology including the isolation and titration of viruses (Busby *et al.*, 1964), investigation of replication cycles (Nathanson 1997) and factors which affect viral growth (Rubin & Fields, 1980; Verdin *et al.*, 1986).

To date there has been little attempt to use such technology to study viral infections in crustaceans primarily because of the lack of suitable cell culture systems. There have only been two reports to date, which describe the culture of MBV (Chen & Kou *et al.*, 1989) and YBV (Lu *et al.*, 1995) in primary cultures of lymphoid tissue in *P. monodon*. As yet, no *in vitro* systems exist for other important crustacean pathogens such as reoviruses, parvo-like viruses or picornoviruses (reviewed by Vega-villasante & Puente, 1993).

Many reoviruses and reo-like viruses (RLVs) have been reported from various crustaceans including the crabs *L. depurator* (Bonami, 1973), *C. mediterraneus* (Mari & Bonami, 1987) and *C. sapidus* (Johnson & Bodammer, 1975; Johnson, 1977) and the prawns *P. japonicus* (Tsing & Bonami, 1987), *P. vannemi* (Krol *et al.*, 1990) and *P. monodon* (Nash *et al.*, 1988). Although high mortality levels have been recorded in animals infected with reovirus or reovirus-like infections (Bonami, 1980; Nash *et al.*, 1988), it is difficult to determine if reovirus is directly responsible for death, acts synergistically with other pathogens or is present as endemic sub clinical infections. Clearly, the isolation and culture of reoviruses is an essential prerequisite for the investigation of pathogenicity and transmissibility of reovirus strains.

Reoviruses from vertebrates have been successfully cultured in a variety of mammalian and avian cell lines (reviewed by Virgin *et al.*, 1997), including bone marrow derived macrophages (Bulow & Klasen, 1983) and peripheral blood monocyte cultures (Hafer, 1984). *In vitro* systems have proved to be excellent models to study reovirus replication and pathogenesis in vertebrate groups (see reviews by Tyler & Fields, 1996; Nathanson 1997) although, invertebrate reoviruses have not yet been successfully cultured. Therefore, the aim of this chapter was to attempt to infect primary cultures of haemocytes (see chapter 5) from the swimming crab, *L. depurator*, with, P virus.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Swimming crabs (*L. depurator*) were collected and maintained as described in chapter 2 (section 2.2.1). Only healthy, male inter moult crabs were selected for bleeding.

6.2.2 Haemocyte culture

Haemolymph was extracted from *L. depurator* as described in Chapter 5, section 5.2.2. The haemocytes were separated on preformed gradients of 50 % percoll (Pharmacia, Uppsala, Sweden) in sterile, filtered 3.2 % NaCl following the procedure described in Söderhäll & Smith (1983).

Hyaline and semi-granular haemocytes were cultured in L15 supplemented with 0.4 M NaCl, 1 % PS (antibiotics) and 10 % FCS at 15º C as described in Chapter 5 (section 5.2.4).

6.2.3 Virus purification

Purified virus samples were prepared as described in Montanie *et al.*, (1983) Virus stock (for *in vitro* infection) was prepared by making a 1:10 dilution of the purified sample in sterile TN buffer (Appendix 1).

6.2.4 *In vitro* infection of *L. depurator* haemocytes.

Hyaline and semi-granular haemocyte cultures were set up in 25 cm^2 flasks as described in Chapter 5 (section 5.2.4) and incubated at 15° C for 24 h. The medium was aspirated after 24 h and replaced with either a final volume of 5 ml of fresh medium (containing 500 µl of TN buffer) (control flasks), 5 ml of medium containing a 10^{-2} dilution of P virus (10^{-2} flasks) or 5 ml of medium containing a 10^{-3} dilution of P virus (10^{-3} flasks). The flasks were incubated for 3, 7 or 14 days and then any haemocytes still attached to the base of the flask were scraped off using a sterile cell scraper. The medium, plus haemocytes, was removed and placed in a sterile tube for further analysis.

6.2.5 Haemocyte counting and viability assays

One millilitre of medium, plus haemocytes, obtained from each flask (see 6.2.5) was used for haemocyte counting and viability assays as described in chapter 5 (section 5.2.4). Haemocytes from a minimum of five flasks were counted for each treatment.

6.2.6 Dot blot hybridisation

One millilitre of medium obtained from each flask (see 6.2.5) was taken and stored at -20° C for use in dot blot hybridisation. Samples were freeze thawed twice before placing 1 µl drops of each sample onto nylon membranes as described in chapter 3 (section 3.2.4). The intensity of

signal produced was used to assess the quantity of virus present in each flask.

6.2.7 Haemocyte morphology

For light microscopy, 0.5 ml of medium obtained from each flask (see 6.2.5) was taken and used for cytospin preparations as described in 2.2.3.

6.2.8 Statistics

Haemocyte numbers are expressed as mean ± standard error of the mean and subjected to square root transformation and two-way ANOVA (Sokal and Rohlf 1981). Haemocyte viability data are expressed as mean % viability ± standard error of the mean. Haemocyte viability data are arcsine transformed and also subjected to two-way ANOVA (Sokal & Rohlf 1981). In both cases, two-way ANOVA was used to determine differences over the culture period and between the treatments at each time interval. A value of $P < 0.05$ was taken as an accepted level of significance.

6.3 RESULTS

6.3.1 Variation in haemocyte number between treatments.

The numbers of hyaline haemocytes varied significantly over the culture period (3 - 14 days) and between treatments (control, 10^{-2} or 10^{-3}) ($P < 0.05$) (Fig 6.1A). In control flasks, hyaline haemocyte number increased from $0.81 \pm 0.13 \times 10^5$ ml^{-1} at 3 days to $2.07 \pm 0.99 \times 10^5$ ml^{-1} after 7 days ($P < 0.05$). After 14 days however, it decreased significantly to 0.32 ±

0.05×10^5 ml^{-1} ($P < 0.05$) (Fig. 6.1A). In 10^{-2} flasks, the hyaline cell number decreased significantly from $1.41 \pm 0.28 \times 10^5$ ml^{-1} at 3 days, to $1.01 \pm 0.17 \times 10^5$ ml^{-1} at 7 days ($P < 0.05$) and $0.47 \pm 0.19 \times 10^5$ ml^{-1} at 14 days post infection ($P < 0.05$) (Fig. 6.1A). Cell number also decreased significantly in 10^{-3} flasks from $1.17 \pm 0.27 \times 10^5 ml^{-1}$ at 3 days, to $0.36 \pm 0.09 \times 10^5 ml^{-1}$ at 7 days and $0.15 \pm 0.07 \times 10^5$ ml-1 at 14 days post infection ($P < 0.05$) (Fig. 6.1A).

Similarly, semi-granular haemocyte numbers also showed significant variation over the culture period of 3 to 14 days ($P < 0.05$), although no significant difference was observed between treatments (control, 10^{-2} or 10^{-3}) at each time interval (Fig 6.1B). Semi-granular haemocytes from control and 10^{-3} flasks exhibited a similar pattern to that observed in control flasks containing hyaline haemocytes. There was significant variation in semi-granular haemocyte numbers from control flasks, haemocyte number increased from 2.08 ± 0.39 ml^{-1} at 3 days to 4.23 ± 0.62 ml^{-1} at 7 days and decreased to 1.94 ± 0.60 ml^{-1} at 14 days post infection ($P < 0.05$) (Fig. 6.1B). In 10^{-3} flasks, haemocyte number increased from 2.64 ± 0.57 ml^{-1} at 3 days to 3.61 ml^{-1} at 7 days and decreased to 0.67 ml^{-1} at 14 days post infection ($P < 0.05$) (Fig. 6.1B). In 10^{-2} flasks, there was a decrease in haemocyte number from 2.27 ± 0.48 ml^{-1} at 3 days to 2.19 ± 0.75 ml^{-1} at 7 days and 1.02 ± 0.44 ml^{-1} at 14 days ($P < 0.05$) (Fig. 6.1B).

6.3.2 Haemocyte viability

Hyaline haemocyte viability did not change significantly over the culture period (3 - 14 days) (Fig 6.2A). In control flasks it ranged from 71.7 ± 5.1 % at 3 days to 87.3± 5.0 % at 14 days post inoculation; in 10^{-2} flasks it ranged from 71.2 ± 6.3 at 3 days to 59.1 ± 15.2 % at 14 days and in 10^{-3}

flasks it ranged from 85.4 ± 3.3 % at 3 days to 70.1 ± 16.1 % at 14 days post inoculation (Fig 6.2A).

Viability of semi-granular haemocytes showed significant variation between treatments at 7 and 14 days ($P < 0.05$) but not at 3 days post inoculation. At 3 days post inoculation, haemocyte viability was 80.3 ± 1.9 % in control flasks, 79.5 ± 6.9 % in 10^{-2} flasks and 87.2 ± 4.1 % in 10^{-3} flasks (Fig. 6.2B). There was a significant reduction in haemocyte viability of semi-granular haemocytes from 10^{-2} flasks at 7 and 14 days post infection compared to control and 10^{-3} flasks ($P < 0.05$) (Fig. 6.2B). At 7 days post infection, cell viability in 10^{-2} flasks was 47. 2 ± 12.5 % compared to 79.5 ± 6.9 % in control flasks and 78.3 ± 3.6 % in 10^{-3} ($P < 0.05$). At 14 days post infection cell viability in 10^{-2} flasks was 52.9 ± 19.7 % compared to 87.2 ± 4.1 % in control flasks and 85.1 ± 3.5 % in 10^{-3} flasks ($P < 0.05$) (Fig. 6.2B).

6.3.3 Dot blot hybridisation

Dot blot hybridisation revealed which flasks contained detectable levels of P virus at 3, 7 or 14 days post infection. A typical dot blot result is shown in Fig 6.3. On this membrane, P virus was detected in samples from flasks containing hyaline and semi granular haemocytes in 10^{-2} dilution and 10^{-3} dilution of virus. Strong signals were obtained at 7 days post inoculation from semi-granular haemocytes in 10^{-2} flasks (Fig 6.3). No virus was detected in control flask 7 (7 days post infection), although weak signals were detected in the other three controls (1, 10 and 16). Data from numerous dot blots indicated that little or no virus was present in hyaline haemocyte control flasks and those flasks containing a 10^{-3} dilution of virus at 3, 7 or 14 days post infection (Table 6.1). In flasks containing a 10^{-2} dilution of virus, signals were visibly more intense

than either control flasks or those containing a 10^{-3} dilution of virus (Table 1). In semi-granular haemocyte cultures, there was also little or no virus present in control flasks at 3, 7 or 14 days post infection (Table 6.2). Signals were more intense in flasks containing a 10^{-2} dilution of virus, particularly at 7 days post infection (Table 6.2, Fig 6.3). In flasks containing a 10^{-3} dilution of virus, signals were also more intense than controls (Table 6.2).

6.3.4 Haemocyte morphology

Under a light microscope, the majority of hyaline haemocytes from control flasks remained intact throughout the culture period (Fig 6.4A). Moreover, haemocytes tended not to aggregate together (Fig 6.4A). In contrast, haemocytes from flasks containing a 10^{-2} or 10^{-3} dilution of virus formed aggregations on the base of culture flasks (Fig 6.4B & C). The cytoplasm of haemocytes from these treatments was often necrotic (Fig 6.4B). In addition, cytoplasm in haemocytes from 10^{-3} flasks appeared vacuolised (Fig 6.4C).

The majority of semi-granular haemocytes from control flasks remained intact throughout the culture period, although they tended to aggregate more than the hyaline haemocytes (Fig 6.4D). The haemocytes from flasks containing 10^{-2} and 10^{-3} dilutions of virus formed similar aggregations to those described above for hyaline haemocytes and appeared necrotic (Fig 6.4E & F). Vacuolisation also occurred in the cytoplasm of haemocytes from the 10^{-3} flasks (Fig. 6.4f).

6.4 DISCUSSION

The *in vitro* propagation of P virus was not successful in this study although P virus had a number of interesting and significant effects on hyaline and semi-granular haemocytes *in vitro*. The addition of P virus (at a dilution of 10^{-2}) produced a significant reduction in hyaline and semi-granular haemocyte numbers and, in semi-granular haemocytes only, this was coupled with a decrease in haemocyte viability. In addition, there were some marked differences in haemocyte morphology, those from flasks containing P virus tended to aggregate in clumps when viewed at the light microscope level. Hyaline and semi-granular haemocytes exhibited cytopathic effects when exposed to P virus *in vitro*. Haemocytes from flasks with a higher concentration of virus (10^{-2}) were often necrotic while those from flasks with a lower concentration (10^{-3}) of virus showed vacuolisation. Similar, vacuolisation was also observed in haemocytes from *L. depurator* inoculated with P virus *in vivo* (see Chapter 2). These effects provide strong evidence that the haemocytes of *L. depurator* are capable of recognising P virus but whether this is the initiation of viral infection or an immunological response is not known.

Dot blot hybridisation, used to determine whether P virus was present at higher levels than the initial inoculum, confirmed the presence of P virus in flasks containing 10^{-2} and 10^{-3} dilutions of virus at higher levels than the dose added. However, low levels of P virus were also observed in some of the control flasks. In Chapter 4, P virus was found in tissue homogenates from natural populations of *L. depurator*, therefore, it is possible some control animals were naturally harbouring P virus infection. If the screening procedure developed in Chapter 4 can be modified to effectively screen haemolymph samples, then presence of

P virus could be determined before cells are taken for *in vitro* investigations. Until this technique is developed, it is impossible to determine whether infection is due to the addition of virus to haemocyte cultures or from replication of virus already present in haemocytes. Consequently, although strong signals were observed in virus infected flasks in this study, indicating that some replication has taken place *in vitro*, without a true negative control *in vitro* infection cannot be substantiated.

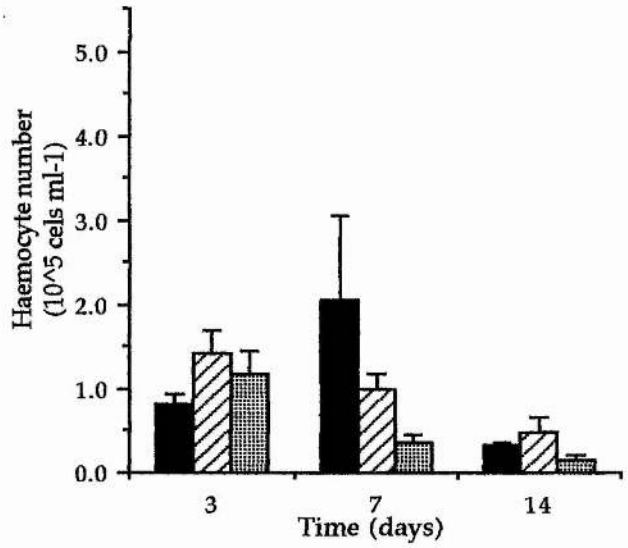
This study has demonstrated that haemocytes can recognise P virus *in vitro* but that *in vitro* infection is complex and difficult to establish. It is well known that viruses frequently grow poorly on initial isolation but adapt on being passaged from culture to culture (Dimmock & Primrose 1987). Therefore, passaging, particularly with cultures that produced strong signals on hybridisation with the P virus gene probe, may increase the possibility of establishing *in vitro* infection. There are numerous other factors which also affect the production of viruses *in vitro* including, titre of infectious virions, cell type, temperature, pH and nutrient levels (Busby *et al.*, 1964). In mammalian reovirus replication (reviewed by Nathanson 1997), pH in particular can affect viral growth *in vitro* because it affects intracellular uncoating, the most critical step in establishment of reovirus infections (Maratos Flier *et al.*, 1986; Sturzenbecker *et al.*, 1987). It is not known how these factors may affect *in vitro* propagation of viruses from invertebrates. Further investigations, using varying titres of virus, pH, temperature and nutrient levels are essential to successfully establish P virus infections *in vitro*.

Figure 6.1 Haemocyte numbers from (A) hyaline and (B) semi-granular cells in flasks containing L15 medium supplemented with 1 % PS (antibiotics), 10 % FCS and 0.4 M NaCl. Haemocyte number was determined at 3, 7 and 14 days following inoculation with fresh buffer (controls), or 10^{-2} or 10^{-3} dilution of virus.

A. Hyaline haemocyte numbers expressed as number of cells $ml^{-1} \times 10^5 \pm$ standard error of the mean. Control flasks (■); flasks containing a 10^{-2} dilution of purified P virus (▨); flasks containing a 10^{-3} dilution of purified P virus (▩). n = 5.

B. Semi-granular haemocyte numbers expressed as number of cells $ml^{-1} \times 10^5 \pm$ standard error of the mean. Control flasks (■); flasks containing a 10^{-2} dilution of purified P virus (▨); flasks containing a 10^{-3} dilution of purified P virus (▩). n = 5.

A. Hyaline haemocytes



B. Semi-granular haemocytes

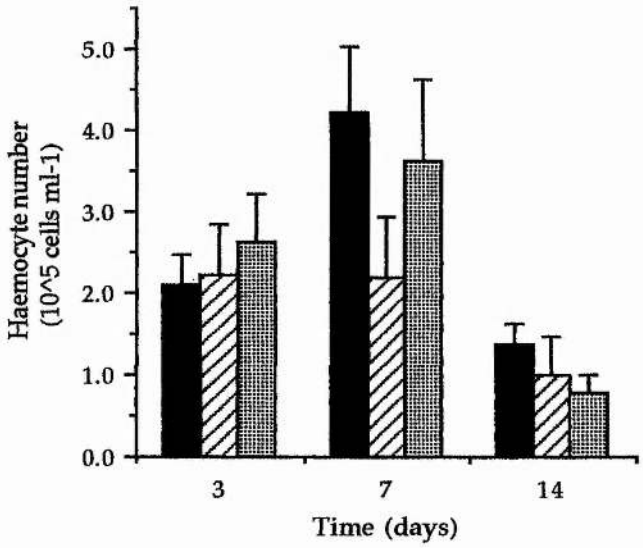
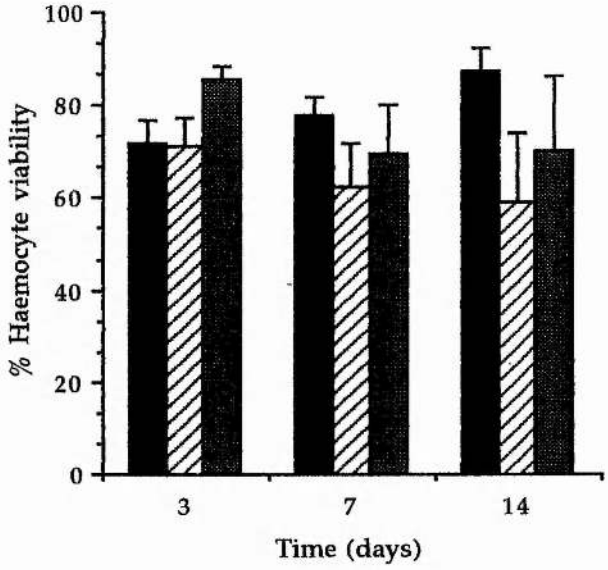


Figure 6.2 Haemocyte viabilities from (A) hyaline and (B) semi-granular haemocytes in flasks containing L15 medium supplemented with 1 % PS, 10 % FCS and 0.4 M NaCl. Haemocyte viability was determined at 3, 7 or 14 days following inoculation with fresh medium containing TN buffer (controls), or 10^{-2} or 10^{-3} dilution of virus.

A. Hyaline haemocyte viability expressed as mean % viability ± standard error of the mean. Control flasks (■); flasks containing a 10^{-2} dilution of purified P virus (▨); flasks containing a 10^{-3} dilution of purified P virus (▩). n = 5.

B. Semi-granular haemocyte viability was expressed as mean % viability ± standard error of the mean. Control flasks (■); flasks containing a 10^{-2} dilution of purified P virus (▨); flasks containing a 10^{-3} dilution of purified P virus (▩). n = 5.

A. Hyaline haemocytes



B. Semi-granular haemocytes

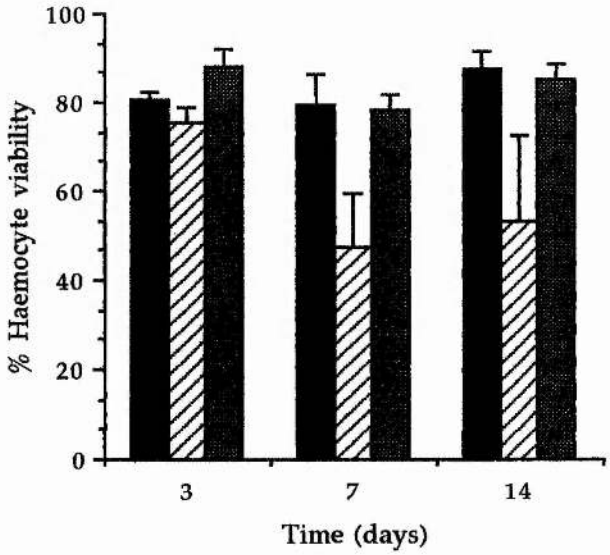


Figure 6.3 Dot blot hybridisation, using E2b probe, on hyaline and semi-granular haemocyte cultures containing L15 medium supplemented with 1 % PS 10 % FCS and 0.4 M NaCl. haemocytes were treated with either fresh medium containing TN buffer (controls); medium containing a 10^{-2} dilution of purified P virus or a 10^{-3} dilution of P virus.

1 = semi-granular haemocytes, 3 days post inoculation, control flasks.

2, 3 = semi-granular haemocytes, 3 days post inoculation, 10^{-2} flasks.

4 = hyaline haemocytes, 3 days post inoculation, 10^{-3} flasks.

5 = semi-granular haemocytes, 3 days post inoculation, 10^{-3} flasks.

6, 10 = hyaline haemocytes, 7 days post inoculation, control flasks.

7, 11 = hyaline haemocytes, 7 days post inoculation, 10^{-2} flasks.

8, 9 = hyaline haemocytes, 7 days post inoculation, 10^{-3} flasks.

12, 13 = semi-granular haemocytes, 7 days post inoculation, 10^{-2} flasks.

14, 15 = semi-granular haemocytes, 7 days post inoculation, 10^{-3} flasks.

16, 17 = semi-granular haemocytes, 7 days post inoculation control flasks.

18 = positive control, pUC 19 plasmid containing insert E2a.

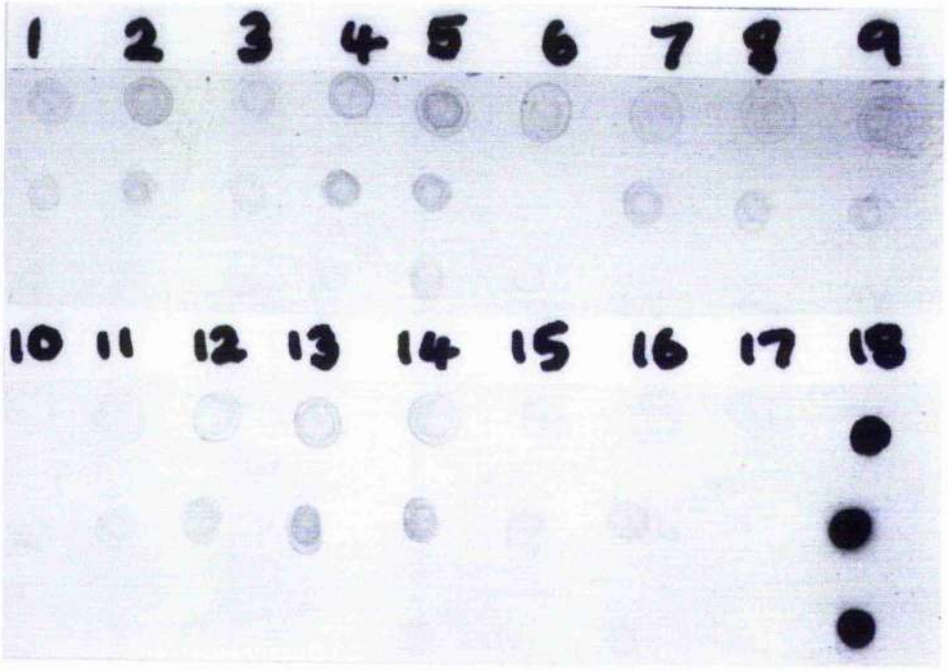

1 2 3 4 5 6 7 8 9
10 11 12 13 14 15 16 17 18

Table 6.1 Signal intensity from dot blot hybridisations of primary hyaline haemocyte cultures inoculated with fresh medium containing TN buffer (Control), 10^{-2} dilution of P virus or 10^{-3} dilution of P virus. The gene probe hybridises to P virus and produces strong signals on nylon membranes. Signal intensity was scored as + = little or no staining present, ++ = clear, positive staining, +++ = strong positive staining. (Fig 6.3 shows a typical dot blot result from which these results were complied).

Table 6.1

DAY	TREATMENT		
	Control	10^{-2}	10^{-3}
3	+	+	+
7	+	++	+
14	+	++	+

Table 6.2 Signal intensity from dot blot hybridisations of primary semi-granular haemocyte cultures inoculated with fresh medium containing TN buffer (Control), 10^{-2} dilution of P virus or 10^{-3} dilution of P virus. The gene probe hybridises to P virus and produces strong signals on nylon membranes. Signal intensity was scored as + = little or no staining present, ++ = clear, positive staining, +++ = strong positive staining. (Fig 6.3 shows a typical dot blot result from which these results were complied).

Table 6.2

DAY	TREATMENT		
	Control	10^{-2}	10^{-3}
3	+	++	+++
7	+	+++	++
14	+	++	++

Figure 6.4 Haemocyte morphology of hyaline haemocytes (A-C) from control (A), 10^{-2} (B) and 10^{-3} (C) flasks and semi-granular haemocytes (D-F) from control (D), 10^{-2} (E) and 10^{-3} (F), 7 days post inoculation.

A. Hyaline haemocytes from control flasks. Haemocytes appear intact with little signs of necrosis and no vacuolisation. Scale bar = 5 μm.

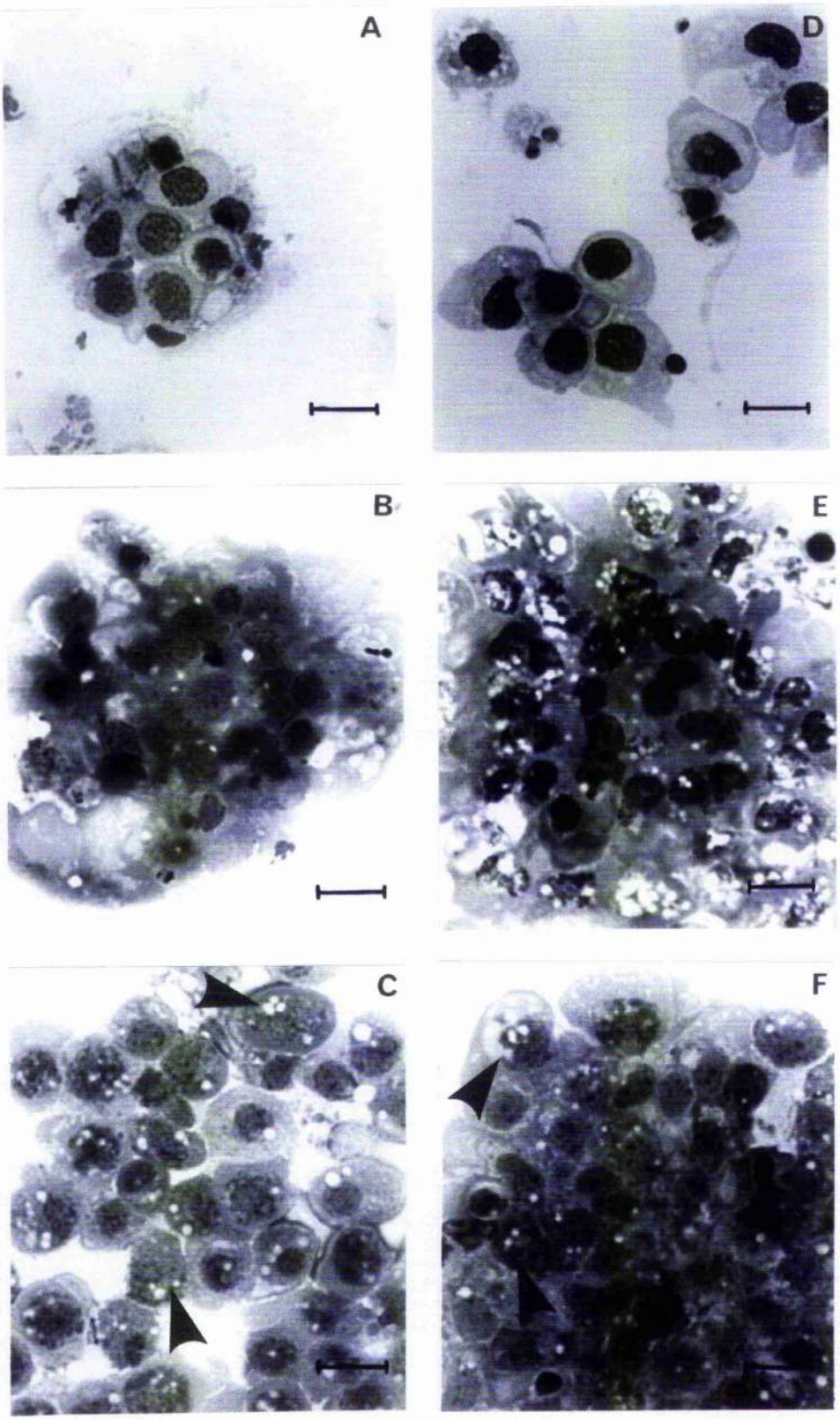
B. Hyaline haemocytes from flasks containing a 10^{-2} dilution of P virus. Haemocytes form large aggregations and appear necrotic. Some haemocytes also appear vacuolised. Scale bar = 5 μm.

C. Hyaline haemocytes from flasks containing a 10^{-3} dilution of P virus. Haemocytes do not appear as necrotic as those from 10^{-2} flasks (see B) however, vacuolisation of the haemocytes is evident (arrows). Scale bar = 5 μm.

D. Semi-granular haemocytes from control flasks. Haemocytes appear intact with little signs of necrosis and no vacuolisation. Scale bar = 5 μm.

E. Semi-granular haemocytes from flasks containing a 10^{-2} dilution of P virus. Haemocytes form large aggregations and appear necrotic. Some haemocytes also appear vacuolised. Scale bar = 5 μm.

F. Semi-granular haemocytes from flasks containing a 10^{-3} dilution of P virus. Haemocytes do not appear as necrotic as those from 10^{-2} flasks (see B) however, vacuolisation of the haemocytes is evident (arrows). Scale bar = 5 μm.


A
D
B
E
C
F

Chapter 7

GENERAL DISCUSSION

This study reports the development of two important techniques, gene probes and haemocyte cultures, that have not been previously available to investigate viral diseases in temperate water marine invertebrates. These techniques were used to investigate numerous aspects of a reovirus infection, P virus, of the swimming crab, *L. depurator*, both *in vivo* and *in vitro*. The construction of the gene probe E2b allowed P virus to be detected in tissue sections and homogenates prepared from infected animals. The use of this gene probe revealed that, not only could virus be experimentally transmitted to *L. depurator* by injection, but that it is present in natural populations of crabs from the North Sea. A seasonal variation in both incidence of P infection and in incubation time was observed, incidence of infection was found to be positively correlated to sea water temperature whereas incubation time was negatively correlated to temperature. This study is the first to use a gene probe to investigate prevalence of a viral infection in a wild population of temperate water crustaceans. Until now, studies have focused on cultured populations of crustaceans, particularly penaeid shrimps, because viral diseases are a major problem in such systems and they are easier to investigate in contained environments (Lightner, 1993).

In vivo, P virus was found to cause marked haemocytopenia in infected *L. depurator* and also a cytopathic effect, producing vacuolisation of haemocytes. Haemocytopenia has been observed in other species of crustaceans that have been antigenically challenged. However, haemocyte numbers usually return to restive levels after 24 hours (Cornick & Stewart 1968; McKay *et al.*, 1969; Smith & Ratcliffe, 1980; Smith & Söderhäll, 1983; Lorenzon *et al.*, 1999). In this study, haemocyte numbers did not recover after 24 h but continued to decline. It has been recently determined that there is a minimum level of circulating

haemocytes below which crustaceans are unable to survive (Lorenzon *et al.*, 1999). This threshold of survival ranges from 29 - 40 % in different crustacean species (Lorenzon *et al.*, 1999). It is likely that the marked haemocytopenia observed in *L. depurator* infected with P virus, particularly in the latter stages of infection, is a major factor in mortality of P-infected *L. depurator*.

Interestingly, haemocytopenia or cytopathic effects are not observed in the haemocytes of *C. maenas*, suggesting that P virus does not infect *C. maenas* haemocytes. Since the initial stage of virus infection requires binding of virus to a recognition site on the host cell, it seems likely that P virus is not capable of binding to *C. maenas* haemocytes. This is an important finding because it offers an opportunity to investigate antiviral mechanisms in invertebrates using a resistant (*C. maenas*) and susceptible host (*L. depurator*). Such investigations have been impossible to date for invertebrates. Hence, antiviral immunity in this group is poorly understood.

In vivo studies also revealed that *L. depurator* infected with P virus often has numerous other infections, including bacterial and amoeboid infections. Reoviruses have been found in association with different viruses in *L. depurator* and other crustaceans, for example, bunyavirus virus (S virus) in *L. depurator* (Bonami 1973) baculoviruses in *P. monodon* (Nash *et al.*, 1988) or rhabdoviruses in *C. sapidus* (Johnson 1984). Whilst it is unclear whether P virus and other reovirus infections are directly responsible for death, act synergistically with other pathogens or are present as endemic sub clinical infections, it is evident that the haemocytopenia observed during P infection will compromise the host immune system and increase susceptibility to other infections.

To address the lack of techniques for *in vitro* studies, a culture system for crustacean haemocytes was developed in this study. A primary culture of hyaline and semi-granular haemocytes was established for haemocytes of both *L. depurator* and *C. maenas*. High cell viability was obtained for at least two weeks and importantly, haemocytes retained their functional capabilities *in vitro*. This is the first crustacean primary haemocyte culture to demonstrate good cell viability and the maintenance of functional activity *in vitro*. There is only one other report to date of haemocyte culture for crustacean haemocytes, from the shrimp *P. monodon* (Ellender *et al.*, 1992). While Ellender *et al.*, (1992) report the maintenance of haemocytes for 3 weeks, they provide no information on viability and functionality over this period. These factors are extremely important because they indicate that the haemocytes are capable of an immune response *in vitro*, which is essential if crustacean haemocyte cultures are to be used for immunological investigations.

In this study, haemocyte proliferation in populations of circulating haemocytes was observed in haemocytes from *L. depurator* that had been maintained, with no treatment, in aquaria for two weeks. To date there are only two other reports of cell proliferation in circulating haemocytes of crustaceans, from penaeid shrimps (Ellender *et al.*, 1992; Sequiera *et al.*, 1996). Ellender et al., (1992) used uptake of BrDU (5-bromo-2'-deoxyuridine), a thymidine analog, to assess haemocyte proliferation in penaeid shrimps. Although they report a 2 % level of proliferation in fresh haemocytes, no data are presented in the paper (Ellender *et al.*, 1992). Sequeria *et al.*, (1996) used flow cytometry to determine levels of proliferation in circulating haemocytes. Flow cytometry is a useful technique because it is possible to analyse individuals and obtain

statistically significant results (Sequeria *et al.*, 1996). The disadvantage of this technique is that it is not possible to directly observe cells. Morphological examination is important to determine whether proliferating haemocytes exhibit different morphology to other haemocytes. Ideally, a combination of techniques, particularly thymidine uptake and flow cytometry, should be used to further investigate haemocyte proliferation in crustaceans.

Haemopoiesis is poorly understood in crustaceans and there are many conflicting hypothesises on the origin and maturation of haemocytes (e.g. Bauchau, 1981; Ghiretti-Magaldi, 1977). The discovery of proliferation of circulating haemocytes is very significant because it may be possible to stimulate cells to divide *in vitro* and establish a crustacean haemocyte cell line. This would be an excellent tool with which to investigate haemopoiesis in crustaceans, indeed pioneering work on mammalian blood cell lineage was only possible through the development of cell lines for T and B lymphocytes (Pluznik and Sachs 1965; Bradley and Metclaf 1966; Choi and Bloom, 1970; Dexter *et al.* 1977, 1979). Preliminary investigations on stimulating haemocytes from *L. depurator* to divide *in vitro* are currently being undertaken (Smith, Hammond & Walton).

Having successfully established a haemocyte culture system for decapod crustaceans and a sensitive methods for detecting P virus, the gene probe E2b, it was possible to begin investigations on P virus infections *in vitro*. P virus certainly produced a number of effects on haemocytes of *L. depurator in vitro*. Haemocyte number and haemocyte viability decreased after addition of P virus and a number of cytopathic effects were observed such as necrosis, pycnosis and vacuolisation. However, during this study it was not possible to

conclusively demonstrate that P virus replicated *in vitro*. It is known that viruses frequently grow poorly on initial isolation but adapt on being passaged from culture to culture (Dimmock & Primrose 1987) and that many factors including titre of infectious virions, cell type, temperature, pH and nutrient levels affect production of virus *in vitro* (Busby *et al.*, 1964). Clearly, these factors require further investigation before P virus infection can be established *in vitro*.

The overall aim of the study was to develop techniques to facilitate the study of viral diseases in crustaceans. Most of them were fulfilled in this investigation. A gene probe was successfully produced and cell culture system established which enabled animals to be screened and hypotheses to be tested. The propagation of P virus was attempted in this study but P infection could not be conclusively confirmed, although a number effects were reported (described above). It was evident from preliminary investigations that a number of modifications could be made to increase possibility of infection that include increasing seeding density of haemocytes, increasing infective dose of virus and passaging virus through numerous cultures.

The most important aim of future work is to further develop the *in vitro* system to successfully propagate P virus. This is important because it will enable host-virus interactions at the cellular level to be investigated. An *in vitro* system will also greatly facilitate immunological investigations, particularly aspects of anti-viral immunity. For example, host defence responses, such as production of reactive oxygen species, could be assessed for anti viral activity *in vitro*. Anti-viral therapeutic compounds, either drugs or anti microbial peptides could also be tested for cytotoxicity. Gene probes will be an important tool, if used in conjunction with *in vitro* systems because they

can detect single copies of genes. Also, they can only hybridise to single strands of target nucleic acid, therefore, if targets are not denatured, using the probe will yield important information on the replicative cycle of viruses. A high intensity signal would be observed when cells are actively replicating.

It would be interesting to determine whether this probe could detect important reoviral pathogens of other crustaceans, apart from *L. depurator*. Reoviruses have been found in commercially important species of crustaceans including and the blue crab, C. *sapidus* (Johnson & Bodammer 1975; Johnson 1977) and the penaeid shrimps *P. japonicus* (Tsing & Bonami 1987), *P. vannemi* (Krol *et al.*, 1990) and *P. monodon* (Nash *et al.*, 1988). If the probe could detect reovirus from these species, it will help to reveal whether there is conservation of genetic material in reoviruses from different species. Such information is important to assess transmissibility of disease for different geographical areas or stocks of crustaceans. In addition, since reoviruses are usually found in association with other pathogens, detection of reovirus by gene probes may be useful as an 'indicator' of a general diseased state in these commercially important species of crustaceans.

A lack of suitable technologies in crustacean virology and immunology has hampered research in these fields to date. The development of sensitive tools to investigate viral disease, particularly gene probes and cell culture systems, have long been recognised by key as essential to advance our understanding of viral disease. Until recent years, study of viral disease has been limited to populations of cultured crustaceans, where disease is readily evident and can produce significant outbreaks. Investigations of such disease has largely been post-mortem and involved characterising the pathogen and tissue histopathology. The

development of gene probes in particular, offer a real opportunity to study viral infections in wild populations of animals and reservoirs of virus in the aquatic environment. The role of marine viruses in the natural environment is only beginning to be fully appreciated because technology is now available to investigate marine virus ecology.

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APPENDICES

APPENDIX 1

TN buffer

	g per l
0.4 M NaCl	23.376
0.02 M Tris-HCl	2.423

pH 7.4, filter (0.2 μm), autoclave

Marine Anti-coagulant

	g per l
0.45 M NaCl	26.298
0.1 M Glucose	18.016
0.03 M tri Sodium citrate	8.942
0.026 M Citric Acid	5.464
0.01 M EDTA	3.722

pH 4.6, filter (0.2 μm)

Davisons Fixative

95 % Ethanol	330 ml
37 % Formaldehyde	220 ml
Glacial acetic acid	115 ml
Distilled water	335 ml

APPENDIX 2

PREPARATION OF 1 % AGAROSE GELS

To a baked beaker add 0.5 g agarose (1%) and 25 ml 0.5X TBE.

Heat and stir until dissolved.

Remove from heat, add remaining 25ml 0.5X TBE.

Stir again (without heat), add 2.5 µl ethidium bromide (stock 0.01g ml^{-1}).

Cool to 50° C (hand hot) and pour onto a clean gel plate, remove air bubbles with a pipette tip.

Mix 10 µl of sample with 1 µl of 10X GLB and 3 µl of Marker with 1 µl of 10X GLB and load (11 µl well)

Run gel @ 80V (constant) for 30-45 mins.

RECIPES

TBE buffer (1 l of 5X stock)

0.45 M Tris-borate		54.0g
0.45 M Boric acid		27.5g
0.5 M EDTA (pH 8.0)		20.0ml
Distilled water	upto	1 l

filter (0.2 µm), autoclave

Gel loading buffer (GLB)

6 X Stock		10 X GLB	
Ficoll 400	1.5 g	6 X GLB	500 µl
0.5 M EDTA	2 ml	Ficoll 400	0.1 g
Bromophenol blue	0.025 g	0.5 M EDTA	100 µl
Xylene Cyanol	0.02 g	Distilled water	200 µl
Distilled water upto	10 ml		

APPENDIX 2 CONT'D

MOLECULAR WEIGHT MARKERS

Marker 1

Lambda DNA (1 mg ml^{-1} stock)	6 µl
Eco RI (5 000 unit stock)	2 µl
Buffer H (Boehringer Mannheim)	2 µl
Distilled water	10 µl

Digest for 2 hrs at 37° C, then add 50 µl of 0.1 X TE buffer and 10 µl of 10 X GLB

Marker 2

Lambda DNA (1 mg ml^{-1} stock)	6 µl
Hind III (5 000 unit stock)	2 µl
Buffer B (Boehringer Mannheim)	2 µl
Distilled water	10 µl

Digest for 2 hrs at 37° C, then add 50 µl of 0.1 X TE buffer and 10 µl of 10 X GLB

Marker 3

First digestion exactly as for marker 2 then:

Product of first digestion	20 µl
Eco RI	2 µl
Buffer H	2 µl
Distilled water	10 µl

Digest for a further 2 hrs at 37° C

APPENDIX 3

RECIPES FOR RNA-DNA HYBRIDISATIONS (dot blot and *in situ* hybridisation).

STOCKSOLUTIONS

All solution to be made up using DEPC-treated water. All glassware must be baked for at least 24 h at 180° C prior to use. Wear gloves throughout. All powdered reagents should be molecular biology grade (RNAse free).

DEPC - TREATED WATER.
Water 1l
DEPC 0.1ml
Leave overnight in a fume hood then autoclave to remove residual DEPC. Store at room temperature for 3 months

20X SSC (1 l)
3.0M NaCl 175.32g
0.3M Na citrate 88.23g
DEPC water 1 l
pH 7.0 filter (0.2 μm), autoclave
Store at room temperature for 3 months

10% N-lauryl sarcosine (50 ml)
10% (w/v) 5.0g
DEPC water 50 ml
filter (0.2 μm)
Store at room temperature for 3 months

20% SDS stock (50 ml)
20% SDS 10.0g
DEPC water 50 ml
filter (0.2 μm)
Store at room temperature for 2 weeks

Maleic Acid buffer (1 l)
0.1M Maleic acid 11.62g
0.15M NaCl 8.72g
DEPC water 1 l
pH 7.5, adjust with solid NaOH; filter (0.2 μm), autoclave
Store at room temperature for 3 months

10% Blocking Stock (100 ml)
10% (w/v) blocking reagent 10.0g
Maleic Acid buffer 100 ml
heat to dissolve, autoclave
Store at 4° C for 1 month

1M Sodium phosphate (200ml)
1 M dibasic Sodium phosphate 21.3 g
DEPC water 150 ml
add powder to water a little at a time, stir vigorously and heat to dissolve
1 M monobasic Sodium phosphate 6.0 g
DEPC water 50 ml
Add monobasic Sodium phosphate to dibasic to obtain pH 7.0, filter (0.2 μm) and autoclave
Store at room temperature for 3 months

10X MOPS (50 ml)
200 mM MOPS 2.1 g
50 mM Sodium acetate 0.26 g
10 mM EDTA 0.19 g
DEPC water 50 ml
pH 7.0 (0.2 μm), filter, autoclave; keep from sunlight
Store at 4° C for 3 months

Buffer 1 (1 l)
100mM Tris 12.11 g
150mM NaCl 8.77 g
Distilled water 1 l
pH 7.5, filter (0.2 μm), autoclave
Store at 4° C for 3 months

Buffer 3 (1 l)
100mM Tris-HCL 12.11g
100mM NaCl 8.77g
50mM $MgCl_2$ 10.17g (optional - increases detection but can produce high background)
Distilled water 1 l
pH 9.5, filter (0.2 μm) autoclave only without $MgCl_2$
Store at 4° C for 3 months

TE buffer (1 l)
100mM Tris 12.11g
10mM EDTA 3.72g
Distilled water 1 l
pH 8.0, filter (0.2 μm), autoclave
Store at 4° C for 3 months

0.5M EDTA (1 l)
EDTA 186.1g
pH 8.0 Stir, add solid NaOH to dissolve, filter (0.2 μm), autoclave
Store at 4° C for 3 months

EXPERIMENTAL SOLUTIONS
PBS (*in situ* only)
Dissolve 1 tablet in 100 ml of DEPC water, filter (0.2 μm), autoclave
Store at 4° C for 3 months

Proteinase K
MAKE UP IMMEDIATELY PRIOR TO USE
Stock = 1 mg ml^{-1} DEPC water. Aliquot and store at -20° C.
Use at final concentration of 0.5μg ml^{-1}
Defrosted stock must be stored at 4° C and used within one day

Denaturing buffer (10 ml)
100% Formamide 5.0 ml
37% formaldehyde 1.62 ml
10X MOPS 1.0 ml
DEPC water 2.38 ml
Store at 4° C for 3 months

High SDS hybridisation buffer (100 ml)

100% formamide	50.0 ml
20X SSC	10.0 ml
1M Sodium phosphate	5.0 ml
10% block	20.0 ml
10%N-larosarcosine	5.0 ml
20 % SDS	7.0 g
DEPC water	up to 100 ml

To the SDS add all solutions EXCEPT FORMAMIDE. Heat to dissolve, autoclave then add formamide

Store at 4° C for 3 months

2X Wash (500 ml)

20X SSC	50.0 ml
20% SDS	2.5 ml
Distilled water	447.5 ml

Store at 4° C for 3 months

0.5X Wash (500 ml)

20X SSC	12.5 ml
20% SDS	2.5 ml
Distilled water	485.0 ml

Store at 4° C for 3 months

0.1X Wash (500 ml)

20X SSC	2.5 ml
20 % SDS	2.5 ml

Store at 4° C for 3 months

Wash buffer (500 ml)

Maleic Acid buffer	498.5 ml
0.3% Tween 20	1.5 ml

Store at 4° C for 3 months

Anti-DIG solution

10 % Blocking solution	20.0 ml
Anti-DIG	4.0 µl

Use anti-DIG at 0.2 µl ml^{-1}

Store at 4° C for 1 day

Colour solution

NBT	45.0 µl
X phosphate	35.0 µl
Buffer 3	10.0 ml

Store at 4° C for 1 day

Development solution (*in situ* only) (10 ml)

Levamisole (0.24 g ml^{-1} stock)	10.0 µl
NBT	45.0 µl
X phosphate	35.0 µl
Buffer 3	10.0 ml

Store at 4° C for 1 day

APPENDIX 4

1. Preparation of Percoll gradients.

C. maenas haemocytes are separated on 60% percoll gradients. *L. depurator* haemocytes are separated on 50% percoll gradients.

Prepare centrifuge tubes by washing in 6 M Urea overnight and thoroughly rinsing in distilled water, allow to drip dry. Use baked glassware throughout. Rinse glassware that has contained percoll ten times in water to remove percoll residues. 10 ml of 50 or 60 % percoll (see below) is placed in each tube and centrifuged at 42 000 g for 20 min at 4° C. preformed gradients can be stored for upto two weeks at 4° C.

3.2 % NaCl Stock		**32 % NaCl Stock**
NaCl	16 g	32 g
Distilled water	500 ml	100 ml

Filter (0.2 μm) and autoclave

For 50% percoll gradients use the following volumes:

Final volume	100ml	200ml	300ml	400ml
Percoll	45.0	90.0	135.0	180.0
32 % NaCl	5.0	10.0	15.0	20.0
3.2 % NaCl	50.0	100.0	150.0	200.0

60% Percoll gradients

Final volume	100ml	200ml	300ml	400ml
Percoll	54.0	108.0	162.0	216.0
32% NaCl	6.0	12.0	18.0	24.0
3.2% NaCl	40.0	80.0	120.0	160.0

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Primary culture of the hyaline haemocytes from marine decapods

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To address the dearth of techniques for medium term primary culture of shellfish blood cells, a simple method has been devised for the maintenance of crab, *Liocarcinus depurator* (L) and *Carcinus maenas* (L), hyaline haemocytes in monolayer culture *in vitro* for a minimum of 14 days. This is based on L15 medium supplemented with 0·4 M NaCl, 10% foetal calf serum and antibiotics. Separated hyaline cells kept in this medium remain ca. 90% viable after 2 days, >80% after 7 days and >70% after 14 days. More importantly, the cells retain defence functionality, as measured by phagocytic uptake of the marine bacterium, *Psychrobacter immobilis*, over the full 14 day incubation period. This method has potential value in a number of applications, particularly for fundamental studies of crustacean cellular immune processes, pathology or ecotoxicology.

Key words: crustacean, haemocytes, cell culture, phagocytosis.

I. Introduction

Mammalian cell and tissue culture techniques have been available since the turn of the century (Harrison, 1907; Carrel, 1912). They are now used routinely to study a variety of cytological phenomena, for example, the cell cycle, leucocyte maturation, virus replication and cellular immune processes (Freshney, 1983). By contrast, the culture of cells from lower vertebrates and invertebrates has received little attention. Accordingly, there is a notable dearth of cell lines from aquatic or marine animals, a situation of considerable importance in aquaculture where there is a great need for established cell lines from commercially important species to expedite disease diagnosis. The first leucocyte cell line from a teleost was not developed until 1979 when Ellender *et al.* produced one from the spleen of the silver perch, *Bairdiella chrysura*. Subsequently, cell lines, mainly from peripheral blood, spleen or kidney, have been established from carp, *Cyprinus carpio* (Faisal & Ahne, 1990), channel catfish, *Ictalurus punctatus* (Lin *et al.*, 1992), spot, *Leiostomus xanthrus* (Sami *et al.*, 1992), black porgy, *Acanthopagrus schlegeli* (Tung *et al.*, 1991) and Japanese eel, *Anguilla japonica* (Chen *et al.*, 1982).

With respect to invertebrates, despite the huge diversity of invertebrate species and their enormous potential as *in vitro* models for biomedicine and

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ecotoxicology as well as in shellfish production, there are relatively few reports of cell culture methodologies. Grace (1962) and Vago & Quiot (1969) were the first to attempt the culture of tissues from invertebrates, focusing mainly on arthropods. Since these pioneering studies, there have been a number of attempts to develop cell, as opposed to tissue, culture techniques for other animal groups, including sponges (Pomponi *et al.*, 1997), molluscs (Domart-Coulon, 1994; Wen *et al.*, 1993; Mortensen & Glette, 1996), shrimps (Ellender *et al.*, 1992; Lu *et al.*, 1995; Loh *et al.*, 1997) and ascidians (Raftos *et al.*, 1990; Rinkevitch & Rabinowitz, 1993; Smith & Peddie *et al.*, 1995). Most species have received attention only because their cells or tissues produce metabolites of possible pharmacological significance (Pomponi *et al.*, 1997) or because the host serves as a vector for insect pathogens (Gong *et al.*, 1997). There seems to have been little attempt to culture marine invertebrate cells for fundamental studies of cell function, cytopathology or pathogen propagation.

As far as crustaceans are concerned, Lu *et al.* (1995) and Loh *et al.* (1997) have described primary culture of lymphoid tissue from penaeid shrimps, which was developed for titration of viral pathogens *in vitro*. To date, however, there has been only one previous report of successful primary culture of haemocytes from decapods (Ellender *et al.*, 1992). In this study, circulating cells from the shrimps, *Penaeus vannemi* or *Penaeus aztectus* were maintained for 3–4 weeks *in vitro* (Ellender *et al.*, 1992). Unfortunately, the authors provided no information about the viability or functionality of the cells over this period. There are many reasons why crustacean haemocytes are difficult to maintain under prolonged culture conditions. There may be a number of distinct cell populations which have varying physiological requirements *in vitro*. They rarely exhibit proliferation *in vitro* or *in vivo*, and they are highly sensitive to non-self materials, frequently undergoing degranulation, clotting or cell aggregation upon exposure to trace amounts of bacterial endotoxin (Smith & Söderhäll, 1986).

Because of the great economic importance of the crustacean seafood industry in many parts of the world, the aim of the present work was to develop a primary, medium to long term culture system for the haemocytes of marine decapod crustaceans. The objective was to maintain high cell viability for a minimum of 14 days and, importantly, to retain functional activity *in vitro*. This will have value for a variety of *in vitro* applications, such as the analysis of the non-specific cellular immune processes, the non-sacrificial quantification of viral pathogens, and the evaluation of toxicological effects on immunocompetent cells.

The swimming crab, *Liocarcinus depurator*, was selected as a suitable model animal for this work. This species is widely distributed throughout Europe (Christiansen, 1969; Moyse & Smaldon, 1990), is of some commercial importance in the Mediterranean, and has been subjected to extensive pathological examination (Bonami *et al.*, 1975; Bonami, 1980). In addition, *L. depurator* is a small animal, easy to keep in aquarium culture, yet contains a large volume of cell rich haemolymph. In common with other brachyurans, this species contains three populations of circulating haemocytes: the hyaline cells, the semigranular cells and the granular cells (Söderhäll & Smith, 1983). As the

semi-granular and granular haemocytes in related crabs are known to be very labile and form only weak attachment to glass or plastic surfaces (Smith & Ratcliffe, 1978), it was decided to focus attention on the hyaline cells. In shore crabs, these are very stable under short term culture (up to 6 h) in simple saline media, attach strongly to glass or plastic surfaces, and readily phagocytose foreign particles *in vitro* (Smith & Ratcliffe, 1978; Söderhäll *et al.*, 1986). The phagocytic capability of the hyaline cells thus provides a simple means of assessing cell functionality over extended culture periods.

II. Materials and Methods

ANIMALS

Specimens of the swimming crab, *L. depurator*, were collected from St Andrews Bay, Scotland in otter trawls and maintained in a flow-through seawater aquarium (salinity=32‰ ± 2, temperature=9 ± 3° C) with constant aeration. They were fed once per week with chopped herring and only healthy, male, intermoult crabs (carapace width=34–54 mm) were selected for experimental purposes. For some experiments (see below), haemocytes were obtained from healthy, adult specimens of the common shore crab, *Carcinus maenas*. These crabs were collected from St Andrews Bay in creels and maintained under aquarium conditions as above.

COLLECTION OF HAEMOLYMPH AND CELL SEPARATION

Haemolymph of *L. depurator* was extracted into ice cold marine anticoagulant as described previously for *Carcinus maenas* (Söderhäll & Smith, 1983). The haemocytes were separated by density gradient centrifugation using a modification of the procedures described in Söderhäll & Smith (1983). Briefly, the cells were separated on preformed gradients of 50% Percoll (Pharmacia, Uppsala, Sweden) in 3·2% NaCl, spun at 3000 × *g* for 10 min at 4° C and the hyaline cell bands removed from the gradients with a sterile plastic pasteur pipette. They were kept on ice for no longer than 15 min before use. With *C. maenas*, haemolymph was harvested and the hyaline cells separated as described in Söderhäll & Smith (1983) and Söderhäll *et al.* (1986).

CULTURE MEDIA

The following commercially available cell culture media were tested for their ability to maintain viability of *L. depurator* haemocytes in vitro: L15, RPMI or MEM (all from Sigma, Poole, Dorset). Each medium was first rendered isosmotic to crab haemolymph by addition of NaCl to a final concentration of 0·4 M. This produced solutions of 925·8 ± 32·8 mOsM kg^{-1}; a value close to the osmolarity of *L. depurator* haemolymph (916 ± 1·8 mOsM kg^{-1}). Osmolality was measured with a Röbling Osmometer (Camlab, Cambridge, U.K.). A 1·0% (final concentration) solution of a commercially available penicillin-streptomycin mix (10,000 units of penicillin and 10 mg of streptomycin per ml in 0·9% NaCl) (Sigma) was added to each medium to minimise bacterial contamination. In addition, to provide a source of putative

growth factors, media were prepared with 0, 10 or 20% (final concentration) foetal calf serum (FCS) (Globepharm, University of Surrey, Surrey, U.K.).

HAEMOCYTE CULTURE

Haemocyte cultures were set up by adding 0·75 ml of separated hyaline cells suspended in Percoll to 5 ml of the test medium in sterile 25 cm^2 flat plastic culture flasks (Corning, High Wycombe, Bucks, U.K.). The flasks were gassed for 5 s with 5% CO_2-air mix (Ham & McKeehan, 1979) and incubated at either 5 or 15° C. For each temperature regime, the medium was changed every 7 days, and cells removed at intervals of 2, 4, 7 or 14 days for viability and phagocytosis assays (see below). To remove cells, the medium was aspirated, replaced with 0·5 ml of sterile 0·5 M NaCl, and the cells gently dislodged with a sterile rubber cell scraper (Sigma, Poole, Dorset, U.K.). A minimum of five animals was used for each temperature level. Viability of the cells was determined at intervals over 14 days by the eosin dye exclusion method (Wilson, 1986). For each experiment, approx 200 cells were counted in duplicate for each crab. A minimum of five crabs was used for each treatment.

To ascertain whether or not the culture method ultimately derived for *L. depurator* hyaline cells had broad applicability to other marine brachyurans, additional cultures were set up with hyaline cells from the shore crab, *C. maenas*. The cells from *C. maenas* were maintained in sterile L15 medium supplemented with 0·4 M NaCl, 1% of the streptomycin-penicillin mix and 10% FCS at 15° C. The cells were harvested after 2, 7 or 14 days and viability assessed as above.

MORPHOLOGY OF CULTURED CELLS

Cytospin preparations of the cultured haemocytes were made by centrifugation for 10 min, $800 \times g$ (20° C) (Cytospin 3, Shandon, Runcorn, Cheshire, U.K.), using approximately 10^6 cells per slide. The haemocytes were fixed and stained using the Romanovsky method (Diff Quik Staining System, Merz und Dade AG, Switzerland). The slides were dried thoroughly in air, mounted in DePeX mounting medium (BDH, Poole, Dorset, U.K.) and the cells photographed using a Leitz Diaplan 20 phase contrast microscope fitted with a Wild Photoautomat MPS45 attachment.

PHAGOCYTOSIS ASSAY

Functional capability of the cultured haemocytes from *L. depurator* was determined at intervals of 2, 4, 7, 9, 11 or 14 days by evaluation of phagocytic vigour *in vitro*. This was assessed by a modification of the procedure described in Smith & Ratcliffe (1978) and Söderhäll *et al.* (1986) using the marine bacterium, *Psychrobacter immobilis* (NCIMB 308), as the challenge particle. This micro-organism was cultured to log phase in marine broth 2216E (Difco, Detroit, Michigan, US), washed and resuspended to a concentration of $2 \times 10^6\ ml^{-1}$ in sterile 3·2% NaCl, as described in Chisholm & Smith (1992). Monolayers of the haemocytes were prepared by culturing cells for 0, 2, 4, 7, 9,

11 or 14 days at 5 or 15° C. At each time point, the cells were removed from the flasks as described above, and 200 μl of each suspension was pipetted onto clean, pyrogen free coverslips. The cells were allowed to attach to the glass surface for 20 min at 20° C and were washed twice with sterile marine saline (MS) (0·5 M NaCl, 11 mM KCl, 12 mM $CaCl_2.6H_2O$, 45 mM Tris-HCl, 26 mM $MgCl_2.6H_2O$, pH 7·4) before being overlaid with 100 μl of the prepared bacterial suspension. The cell-bacteria mixtures were incubated in a moist chamber for 3 h at 20° C (Smith & Ratcliffe, 1978). They were then washed thoroughly with sterile MS to remove unattached bacteria and finally fixed for 20 min in 10% formaldehyde in seawater. The monolayers were scrutinised under phase contrast optics and the number of cells containing one or more intracellular bacteria (assessed using the criteria given in Smith & Ratcliffe, 1978) determined from a minimum of 100 cells per coverslip. Duplicate coverslips were counted for each time period.

STATISTICAL ANALYSIS

All viability data are expressed as mean % viability ± standard error of the mean. Comparisons of cell viability in various media and at different supplement levels were performed using ANOVA on arcsine transformed data (Sokal & Rohlf, 1981). The effect of temperature on cell viability and comparisons between species were analysed using student's *t*-tests on paired or unpaired, arcsine transformed data where appropriate (Sokal & Rohlf, 1981).

Phagocytosis data are expressed as mean % phagocytosis ± standard error of the mean. Phagocytic uptake of bacteria by cultured haemocytes was compared to uptake in freshly extracted haemocytes using student's *t*-tests on unpaired, arcsine transformed data (Sokal & Rohlf, 1981).

III. Results

MORPHOLOGY OF CULTURED HAEMOCYTES

Large numbers of hyaline haemocytes from *L. depurator* attached to the base of the flasks to form monolayers (Fig. 1A). Similar attachment was seen with hyaline cells from *C. maenas*, although the haemocytes from this crab tended to spread more than those from *L. depurator* (Fig. 1B). There was a slight, but not significant, decrease in the number of attached cells by day 14, but, importantly, cytospin preparations of the cultured haemocytes showed that cells were intact, with no sign of necrosis, pycnosis or contamination (Fig. 1C & D).

HAEMOCYTE CULTURE

As regards cell viability, the haemocytes from *L. depurator* survived but did not grow, in each of the three culture media tested (Fig. 2). After 2 days, cell viability was 70·1 ± 5·8% in L15, 51·9 ± 3·6% in RPMI and 49·3 ± 12·4% in MEM (Fig. 2). However, over the next three days viability of the cells in RPMI or MEM fell dramatically to 16·7 ± 4·7% and 19·2 ± 4·2% respectively. This was significantly lower than cell viability in L15 medium (72·7 ± 5·2%) over

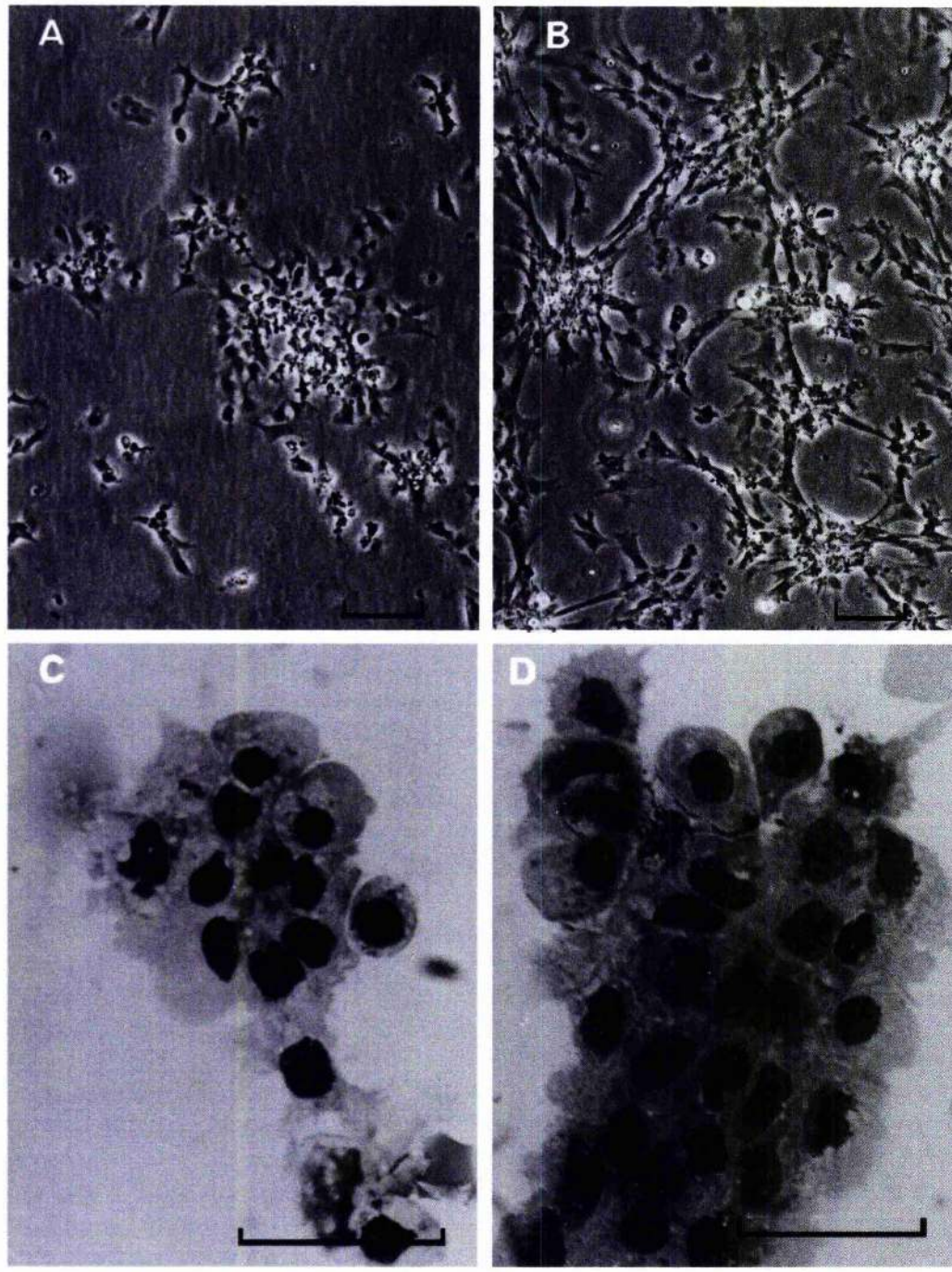

Fig. 1. Appearance of cultured hyaline haemocytes from decapod crustaceans. (A and B) Hyaline haemocytes from *L. depurator* (A) and *C. maenas* (B) after 2 days *in vitro*. Phase contrast optics. Scale bar = 20 μm. (C and D) Cytospin preparation of hyaline haemocytes from *L. depurator* (C) and *C. maenas* (D) after 7 days *in vitro*. Romanovsky stain. Scale bar = 10 μm.

the same time (5 days) ($P<0{\cdot}01$). By 7 days, mean cell viability was $63{\cdot}1 \pm 7{\cdot}94\%$ in L15 but only $9{\cdot}9 \pm 4{\cdot}19\%$ in RPMI and 0·0% in MEM (Fig. 2).

Inclusion of FCS in the salt amended L15 medium significantly promoted viability of the cultured haemocytes. After 2 days, cell viability increased from

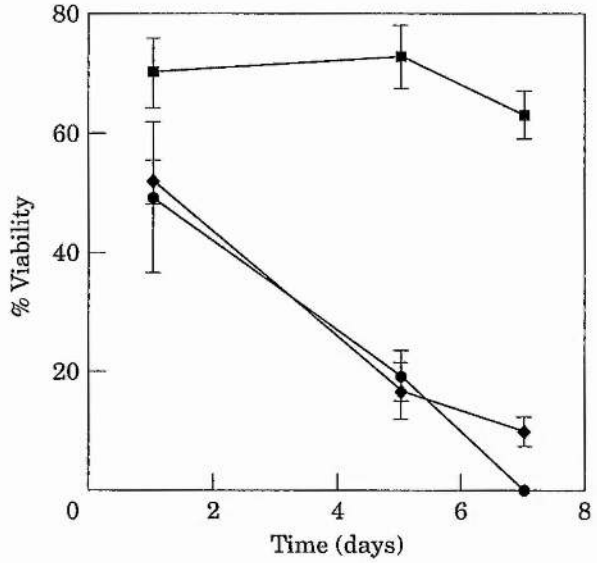

Fig. 2. Survival of hyaline haemocytes from *L. depurator* in different media *in vitro*. L15 (-■-), RPMI 1640 (-◆-) or MEM (-●-). All were supplemented with sterile 0·4 M NaCl and 1% antibiotics (final concentrations). Values given are means ± SE (n=5).

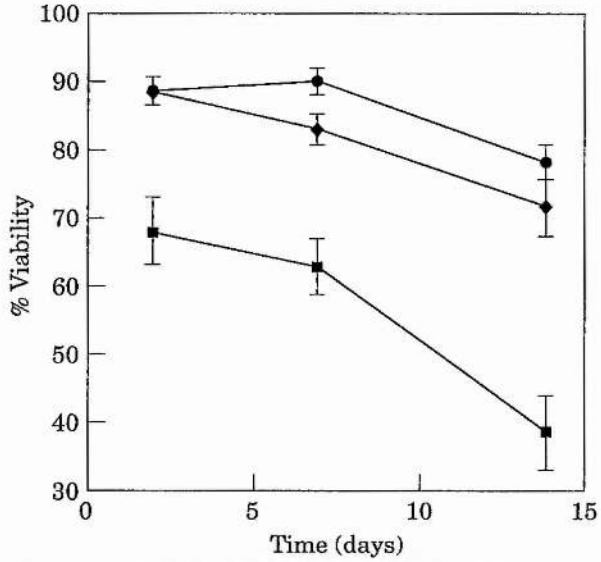

Fig. 3. Viability of cultured hyaline haemocytes from *L. depurator* in 0 (-■-), 10 (-◆-) or 20% (-●-) FCS. The culture medium was L15 medium supplemented with sterile 0·4 M NaCl and 1% antibiotics (final concentrations). Values are means ± SE (n=5).

68·2 ± 4·7% in unsupplemented media to 88·8 ± 2·3% with a 10% FCS supplement and 88·9 ± 2·2% with a 20% FCS supplement (Fig. 3). Importantly, inclusion of FCS prolonged cell survival over the following 12 days so that by day 14, overall viability was 39·1 ± 11·3% without FCS, but 72·0 ± 8·2% in 10% FCS (P<0·01) and 78·6 ± 5·2% in 20% FCS (P<0·01) (Figure 3). There was no significant difference in cell viability between 10 and 20% FCS, but as cell

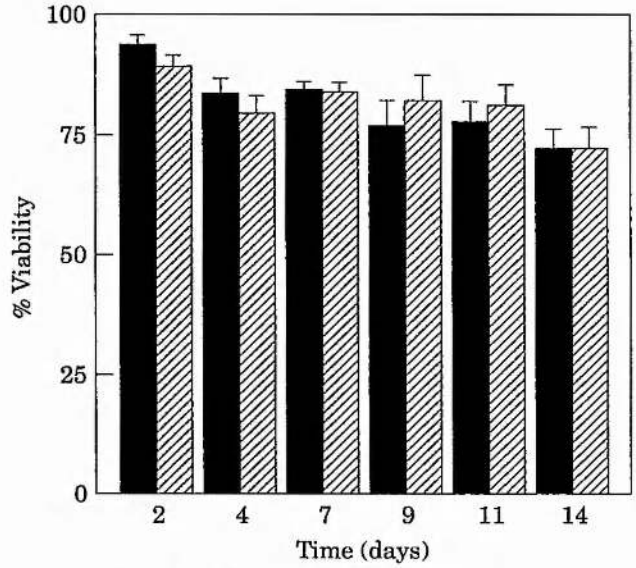

Fig. 4. Survival of cultured hyaline haemocytes from *L. depurator* at different temperatures. The haemocytes were cultured at 5 (■) or 15° C (▨) in L15 medium containing sterile 0·4 M NaCl and 1% antibiotics (final concentrations). Values are means ± SE (n=5).

clumping sometimes occurred when a supplement of 20% FCS was used, all subsequent experiments used only 10% FCS.

EFFECT OF TEMPERATURE

Experiments to investigate the effect of incubation temperature on survival of *L. depurator* hyaline haemocytes *in vitro* revealed that cell viability remained high after 14 days in culture at both temperatures tested. At 2 days, viability was 92·9 ± 1·4% at 5° C and 88·8 ± 2·2% at 15° C; at 7 days it was 83·9 ± 2·2% at 5° C and 83·5 ± 2·0% at 15° C; and at 14 days was 71·8 ± 8·5% at 5° C and 72·0 ± 8·2% at 15° C (Fig. 4). There was no significant difference between the values obtained for the two temperatures at each time point.

COMPARISON BETWEEN SPECIES

Having established good viability of *L. depurator* hyaline cells in L15 medium containing 0·4 M NaCl, antibiotics and 10% FCS, this medium was used to evaluate survival of separated hyaline cells from *C. maenas* at 15° C. Eosin Y exclusion measurements showed that viability of the *C. maenas* cells was 84·3 ± 4·3% after 2 days in culture, 88·1 ± 10·3% after 7 days and 84·2 ± 5·25% after 14 days. These values compare favourably with survival rates for *L. depurator* cells (above).

PHAGOCYTOSIS ASSAY

Hyaline haemocytes from *L. depurator* have the ability to phagocytose the marine bacterium, *P. immobilis*. The mean percentage uptake of this

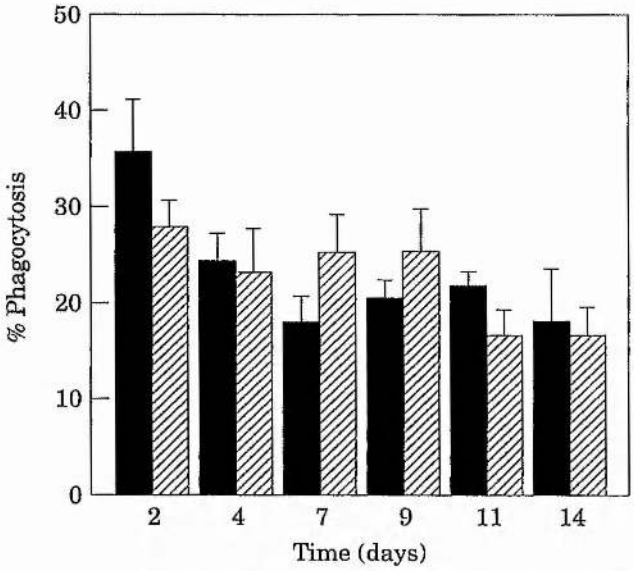

Fig. 5. Phagocytic uptake of the marine bacterium, *Psychrobacter immobilis in vitro* by cultured hyline cells from *L. depurator* at 5 (■) or 15° C (▨). The haemocytes were cultured in L15 medium containing sterile 0·4 M NaCl and 1% antibiotics (final concentrations). Values are means ± SE (n=5).

bacterium in freshly extracted hyaline haemocytes was 18·8 ± 4·9%. Cultured hyaline haemocytes from *L. depurator* were found to retain their ability to phagocytose the bacterium, *P. immobilis*, over the full 14 day culture period (Fig. 5). Cells cultured for 2 days showed considerably elevated levels of uptake, with 35·7 ± 5·5% of those cells previously maintained at 5° C found to contain one or more bacteria, and 27·9 ± 2·8% of those cells cultured at 15° C seen to enclose bacteria (Fig. 5), both values significantly higher than the levels of ingestion observed for freshly extracted phagocytes (P<0·01), but not significantly different from each other. Phagocytic rates declined to around 25% with the 4, 7, and 9 day old cells, although the 15° C incubated cells tended to show similar or slightly higher levels of uptake than the 5° C cells (Fig. 5). Phagocytic rates remained close to ca 17% with the 11 and 14 day old cells, with the mean uptake by 14 day old cells found to be 18·2 ± 5·5% at 5° C and and 16·6 ± 3·0% at 15° C (Fig. 5). Values from day 4 to day 14 were not significantly different to each other or to the level of uptake recorded for freshly extracted haemocytes.

IV. Discussion

This paper describes a method for the *in vitro* culture of the hyaline haemocytes from *L. depurator* and *C. maenas* for a medium term period of 14 days. This method is based on salt amended L15 medium supplemented with antibiotics and 10% FCS. Crucially, this medium maintains high cell viability for both species over the full incubation period, and the cells retain their ability to phagocytose bacteria *in vitro*. To the best of our knowledge, this is

the first report of a primary culture system for decapod crustaceans which favours both survival and functional activity of circulating blood cells for an extended time.

L15, the most suitable medium for the culture of the haemocytes in this study, has also been used successfully for the culture of shrimp nerve, lymphoid or ovary tissues (Nadala *et al.*, 1993). By contrast, RPMI 1640 medium has been preferred for culture of ascidian lymphoid cells (Raftos *et al.*, 1990; Rinkevitch & Rabinowitz, 1993; Smith & Peddie, 1995; Peddie *et al.*, 1995). The culture system used in the present study proved effective in keeping growth of contaminant micro-organisms in check. Other workers, notably, Ellender *et al.* (1992) and Pomponi *et al.* (1997) have experienced serious problems of high levels of contamination in some of their cultures. Not only is it essential to avoid contamination in cell or tissue cultures over prolonged periods to ensure that the cells of interest are not overgrown, but also, for immunological analysis, it is vital to ensure that the cell's environment remains as near endotoxin-free as possible. As the cells did not loose their ability to phagocytose bacteria *in vitro*, it is likely that they had not been 'spontaneously' activated by non-self materials during the culture period.

The present study also differs from earlier reports in incubating the cell cultures at 5 and 15° C, temperatures much lower than the optimal temperatures (25–32° C) found for shrimp cells by Ellender *et al.* (1992). These temperatures (5 and 15° C) were selected as experimental temperatures in the present study because *L. depurator* is a thermo-conforming invertebrate, which on account of its shallow water epibenthic habit, routinely encounters environmental temperatures ranging from 3° C to 18° C (Hayward, 1990). For specimens living in the eastern North Sea, typical winter temperatures are around 5° C while summer temperatures in inshore waters average ca. 15° C (Hayward, 1990). Thus 5 and 15° C are the normal seasonal temperatures encountered by *L. depurator*. In the present study no significant difference was observed between the viability of the haemocytes incubated at 5° C and 15° C, showing that the hyaline cells of *L. depurator* have considerable plasticity in temperature tolerance *in vitro*. Such plasticity has been observed in other cold water species, for example, salmonid cells will grow well at 20° C, tolerate 4° C but die at 26–37° C (Wolf, 1979). This indicates that the optimal incubation temperature for cell culture of poikilothermic animals varies from species to species in accordance with the normal environmental temperature of the host.

L. depurator cells showed good survival and phagocytic responsiveness following incubation at 5 or 15° C. Most previous physiological *in vitro* studies of temperate water marine crustacean blood cells have been carried out at 15 or 20° C (Smith & Söderhäll, 1983; Chisholm & Smith, 1992; Bell & Smith, 1993) and at this temperature uptake of bacteria has been found to be ca. 20%, a value close to that obtained with freshly collected haemocytes from *L. depurator* in the present investigation. No attempt was made to measure uptake of bacteria by cultured hyaline cells from *C. maenas* in this study, although observations revealed that these cells also retain phagocytic capability, even after 14 days *in vitro*. Interestingly, phagocytosis by 2 day cultured

L. depurator haemocytes was found to be higher than freshly collected haemocytes. One explanation for this phenomenon is that there was some pre-selection of phagocytic cells during the first 48 h *in vitro*, producing apparently greater rates of uptake than uncultured cells. Certainly, there was some loss of adherent haemocytes over the 14 day culture period, and this was most marked during the first 48 h (data not shown), so it is possible that the weakly adherent cells are not phagocytically capable; a hypothesis consistent with the suggestion of Thornqvist *et al.* (1994) that adhesion molecules function as opsonins in crustaceans.

An incubation period of 14 days represents a medium term primary culture. Longer term cultures are routine with mammalian cells, and, with ascidians, Rinkevitch & Rabinovitch (1993) have managed to keep lymphoid cells alive for up to 90 days. In the present study, no attempt was made to maintain the cells for longer periods, although preliminary experiments tried to continue culturing the cells for 21 days and indicated that good cell viability could be maintained for longer. What is arguably more important than extended cell survival times, is the ability to promote mitosis within the cultured cell population as a pre-requisite to establishing a continuous cell line. Such cell lines appear to be particularly difficult to obtain from invertebrates because of problems associated with authentication and mitotic stimulation (Freshney, 1983; Pomponi *et al.*, 1997). Indeed, some reports of cell lines from marine invertebrates have actually turned out to have been fungal or protozoan contaminants (Pomponi *et al.*, 1997). To date, there have been no convincing reports that the circulating blood cells of brachyurans undergo mitosis in the haemocoel, although Ellender *et al.* (1992) and Sequeira *et al.* (1996) have observed low levels (1–2%) of haemocyte proliferation in penaeid shrimps. With ascidians, Rinkevitch & Rabinowitz (1993) noted that blood cells from *Botryllus schlosseri* proliferate *in vitro* and that the new cells remain viable over 10 plating cycles. More recently, Peddie *et al.* (1995) found that the blood cells from *Ciona intestinalis* respond positively to treatment with concanavalin A, phytohaemagglutinin or lipopolysaccharide, so it may be possible to stimulate crustacean cells to divide in culture by expeditious use of such mitogens. Preliminary studies of the proliferative capability of crab blood cells *in vitro* are currently being undertaken (Walton, Smith and Hammond).

To conclude, the present study goes a long way to address the problem of the lack of medium term culture technologies for the blood cells of marine invertebrate animals. The above system is simple to set up, inexpensive to maintain and requires minimal attention once established. It can be used to investigate several aspects of cellular immunity, pathology and ecotoxicology in marine crustaceans. Such information is particularly important to the growing aquaculture industry, where there is a distinct need for methods of disease diagnosis, disease control and non-sacrificial evaluation of the effect of environmental quality on health.

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