Advances in Crustacean Cell Culture

Thesis submitted by

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ABSTRACT

In 2006, for the first time in history, more seafood was produced from aquaculture raised animals than wild caught fisheries. However, crustacean aquaculture continues to be plagued by disease, due to the lack of sensitive investigative methods that assist diagnostics and pathogen control. Prevention and control of diseases are an absolute priority for the durability of the crustacean aquaculture industry.

The development of a permanent *in vitro* model for crustacean species is imperative. The research presented herein attempted to utilise novel modern technologies to develop the first continuous cell line from crustacea. The first investigation involved assessing methodologies that explored the existence of crustacean components in hybridised cell lines, which were developed by combining crustacean cells with immortal fish cells (Chapter 4). The methodologies involved cellular assessment from genomic and protein approaches, along with exploration of viral susceptibility of the cells. Using PCR, no crustacean 18S rRNA genes or haemocyanin genes were found in any of the seven hybrid cell lines. No crustacean proteins were detected, nor did any viral amplification occur when hybrid cells were inoculated with two crustacean viruses, indicating that these hybrid cell populations were not suitable for crustacean virological studies (Chapter 5).

The second investigation involved optimisation of *in vitro* methods using the Australian freshwater crayfish, *Cherax quadricarinatus* (Chapters 6 and 7). The approach included scientifically optimising the culture medium by comparing cell proliferation with three different cell culture media and 25 different media supplements. Leibovitz-15 medium, along with supplemental iron, copper, foetal bovine serum, and non-essential amino acids, was found to significantly increase cell proliferation ($F = 6.231$, df = 1,19, $p < 0.05$) and also augment the longevity of cells *in vitro*.

While spontaneous transformation of somatic cells can occur, transgenesis often expedites immortality. Therefore, the third investigation explored the transfection of

C. quadricarinatus primary cells (Chapter 8). A lipofection reagent was used to introduce an observable green fluorescent protein vector into the cytoplasm of the cells. Transfection of the cells was then attempted using human oncogenes.

Papillomaviruses are non-enveloped, double-stranded DNA tumour viruses that play a critical role in the formation of human anogenital cancer. Early studies have demonstrated that the human papillomavirus-expressed E6 and E7 proteins function concomitantly to disrupt the p53 and Rb tumour suppressor genes, regulators of the cell-cycle checkpoints at the first gap (G_1) phase of the cell cycle. To help *C. quadricarinatus* cells pass through the G_1 phase and enter the DNA synthesis stage of the cell cycle, HPV E6 and E7 genes were transfected into the *C. quadricarinatus* cells. Successful transfection was demonstrated by the presence of oncogene mRNA by RT-PCR. At day 150, transfected cells remained viable, although cell proliferation was stagnant. It may be that, although transfection of the oncogenes was successful, no proliferation of the *C. quadricarinatus* cells was evident, due to a lack of telomere maintenance.

Overall, attempts to create a crustacean cell line remain elusive. Although the end product of a permanent cell line has not been forthcoming, this research made successful advancements in crustacean cell culture methodologies and explored new techniques and technologies that may assist eventual immortalisation.

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LIST OF ABBREVIATIONS

2-ME	2-mercaptoethanol
A_{260}	Absorbance at 260nm
A_{280}	Absorbance at 280nm
ABARE	Australian Bureau of Agricultural and Resource Economics
ADS	Appendage deformity syndrome
ANOVA	Analysis of variance
BF-2	Bluegill fry
bFGF	Basic fibroblast growth factor
BLAST	Basic local alignment search tool
BrdU	5-bromo-2'-deoxyuridine
CMRL	Connaught Medical Research Laboratories
CPE	Cytopathic effect
DEAE	N,N-diethylaminoethyl
DMEM	Dubecco's minimum essential medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E6	Human papillomavirus early gene 6
E7	Human papillomavirus early gene 7
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
EPC	Epithelioma papulosum cyprinid
FAO	Food and Agriculture Organisation
FBS	Foetal bovine serum
FCS	Foetal calf serum
FHM	Fathead minnow
GAMA	Gamma -amino butyric acid
GAV	Gill associated virus
GFP	Green fluorescence protein
H&E	Haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid

HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HPLV	Hepatopancreatic parvo-like virus
HPV	Human papillomavirus
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
IGF	Insulin growth factor
L-15	Leibovitz 15 culture medium
LO	Lymphoid organ
LTR	Long terminal repeat
M199	Medium 199
MAb	Monoclonal antibody
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
NCTC	National Centre for Tissue Culture
p53	Protein 53
PAb	Polyclonal antibody
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
PRBV	Penaeid rod-shaped baculovirus
Rb	Retinoblastoma
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SF-9	*Spodoptera frugiperda* pupal ovarian tissue
SV40	Simian virus 40
TC-100	Tissue culture medium 100
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TRAP	Telomeric repeat amplification protocol
WSSV	Whitespot syndrome virus
WST-1	Water soluble tetrazolium salt
YHV	Yellowhead virus

CHAPTER 1
INTRODUCTION

The global demand for aquatic food products is increasing. Production from capture fisheries has levelled off and most of the main fishing areas have reached their maximum potential (FAO, 2006). Therefore, seafood product from capture fisheries will not be able to meet the growing global demand for aquatic food. Aquaculture continues to make a significant contribution to this increasing demand. Currently, one billion people are dependent on aquatic animals as the principal source of animal protein (FAO, 2006). However, disease outbreaks have been increasingly recognised as a significant constraint on aquaculture production and trade, affecting the economic development of the industry in many countries.

Crustaceans represent one of the most economically important global aquaculture sectors, producing in excess of $10 billion USD annually (Johnson *et al.*, 2008). Like other aquatic animals, crustaceans are susceptible to a wide variety of pathogens, including viruses, bacteria, fungi, and protozoa. During the last decade, disease was considered to be the most limiting factor for expansion of crustacean culture (Lin, 1995). In the mid 1990s, it was estimated that around 40% of worldwide prawn production, representing a value of over $3 billion USD, was lost due to diseases (Lundin, 1996). The main contributors to these losses were viruses (Lotz, 1997).

The stresses and close proximity of individuals under intensive aquaculture favour the spread of disease. However, modern practices for finfish aquaculture include highly effective routine vaccination against multiple pathogens, which has dramatically reduced the impact of disease (Johnson *et al.*, 2008). These practices are effective, because teleost fish have adaptive immune responses and immune memory involving B cells and T cells, antibody and phagocytic cells. Understanding these immune mechanisms and how the pathogens interact at the cellular level has allowed aquatic animal health scientists to develop successful vaccines (Sommerset *et al.*, 2005). On the other hand, invertebrates, such as crustaceans, rely solely on an innate immune

system characterised by generalised immune responses. The responses involve an array of pattern recognition receptors that interact with serine proteases to initiate encapsulation, phagocytosis, and an antimicrobial cascade based on the phenoloxidase system (Söderhall and Cerenius, 1998). No recognition system for viral infections has been documented. These animals are therefore highly susceptible to viral infections, which often result in mortality.

However, there is some recent evidence of specific immune memory in crustaceans (Johnson *et al.*, 2008). To assist this understanding, and ultimately control viral disease outbreaks in crustacea, the study of these diseases must go beyond their basic description and identification, and the tools to investigate outbreaks must be developed. Given the breakthroughs obtained in human and veterinary health, the achievement of such measures greatly depends on the development of crustacean cell cultures that permit *in vitro* cultivation of the intracellular pathogenic agents. Such cell cultures could help us to determine and understand the host-pathogen relationship at the cellular and molecular level, as well as assisting us to understand immune functions.

Cell culture can be defined as the maintenance or growth of cells in a nutrient fluid following removal from an organism's body. The concept of cell culture is not new, but began over a hundred and twenty years ago, when in 1885 Wilhelm Roux discovered that chicken embryonic cells could survive outside the animal body (Freshney, 2005). Nearly sixty years later, the study of malignancy *in vitro* (Earle, 1943) led to the development of the first continuous cell line. Today, human cell cultures are powerful and important tools that are used to address many fundamental questions in biology and medicine. A large variety of cell lines exist for many vertebrate species. In contrast, *in vitro* models for invertebrate species, which make up 95% of the animal kingdom (Ruppert and Barnes, 1994), are far less advanced. Since the development of the first invertebrate cell line in 1961 (Grace, 1962), continuous cell lines have been established from more than 100 species of insects (Lynn, 1999). However, no permanent cell culture system exists for any aquatic invertebrate.

Development of a cell line provides many advantages. Unlike virology of aquatic vertebrates, crustacean virology is still in its infancy, and the major goal of cell line development has been to improve knowledge of disease-causing agents. *In vivo* models have limitations for studying disease, and isolation of pathogens *in vivo* is difficult. The production of standardised *in vitro* systems provides a means to study the effects and mechanisms of pathogen invasion and provides a medium for isolation of infectious pathogens. *In vitro* systems also allow the study of cellular biochemistry and can facilitate studies of ecotoxicology (Freshney, 2005). Once cell lines have been established, they reduce the need for animal experimentation, which precludes legal, moral and ethical problems, as cells can be continually cultivated without the need to sacrifice whole organisms. Cell cultures also allow the culture of a single virus, instead of the mixed populations of viruses that are worked with in current bioassay systems (Owens, 1997).

Most of the crustacean species utilised in aquaculture come from the order Decapoda, of which Penaeidea (prawns), Astacidea (lobsters, crayfish), and Brachyura (true crabs) are representative orders. Penaeid species compose the largest group of crustaceans in aquaculture, with global production of approximately 3.16 million mt (FAO, 2006). Therefore, the penaeid species have been the primary focus of cell culture efforts during the past two decades. Chen *et al.,* (1986) was first to report a short term tissue culture of *Penaeus monodon*, with a further nine other penaeid spp. undergoing recent cell culturing efforts. Primary cultures obtained from various penaeid organs are reported with increasing frequency (Wang *et al.*, 2000; Mulford *et al.*, 2001; Lyons-Alcantara *et al.*, 2002; Assavalapsakul *et al.*, 2003; Chen-Lei *et al.*, 2003; Gao *et al.*, 2003; Maeda *et al.*, 2003; Lang *et al.*, 2004; Maeda *et al.*, 2004; Jiang *et al.*, 2005). These studies represent the first steps towards the establishment of cell lines and they provide useful information concerning the most suitable cell culture conditions involved in the survival and proliferative capacity of the various tissues (Toullec, 1999). Examination of the literature on establishment of crustacean and other invertebrate cell lines, as well as on recent advances in technology, identified a number of novel approaches for further investigation, including (1) cell hybridisation, (2) comparison of culture media supplementation

using cell proliferation assays, and (3) cell transformation by transfecting cells with oncogenes. The research presented in this thesis investigated these approaches with the goal of eventually establishing a permanent crustacean cell line.

CHAPTER 2
REVIEW OF LITERATURE

2.1 INTRODUCTION

Cell culture, the maintenance or growth of cells in a nutrient fluid following removal from an organism's body, started over 120 years ago. Harrison (1907) maintained embryonic frog neural crest fragments in a culture fluid for several weeks and is considered the "father" of cell culture (Mothersill and Austin, 2000). Harrison's techniques initiated a wave of interest in the cultivation of animal tissue *in vitro*. Burrows (1910) further advanced the proliferation of chicken embryonic cells in culture and was the first to describe the process of mitosis in detail. Another significant milestone was the development of the first viral vaccine (Vaccinia) from cell culture (Carrel and Rivers, 1927). Following on from this, the role of malignancy was studied *in vitro* by Earle *et al.,* (1943), which led to the development of the first continuous cell line. Earle's demonstration that human tumours could give rise to continuous cell lines increased interest in the development of human cell culture. Today, human cell cultures are a powerful and important tool that has been utilised to address many fundamental questions in biology and medicine. Recent advances have made it possible to use cultured human cells for therapeutic use. Cultured cells have been used to reconstruct skin, bone, and other tissues, including the endothelium (Freshney, 2005).

Today, a variety of cell lines exist for many vertebrate species. In contrast, *in vitro* models for invertebrate species, which make up 95% of the animal kingdom (Ruppert and Barnes, 1994), are far less advanced. Significant advances in invertebrate cell culture were made in the 1950s and 1960s, largely due to the availability of antibiotics and increasing knowledge of biochemistry (Mothersill and Austin, 2000). In 1961, Wyatt analysed insect haemolymph composition and designed a cell culture medium that showed promising results in enabling the survival of insect cells. Grace (1962) made some additions to Wyatt's culture medium and, through a technique described as "organised

neglect," developed the first continuous invertebrate cell line from the gum moth, *Antheraea eucalypti*. Since then, over 400 continuous cell lines have been established from more than 100 species of insects (Lynn, 1999). Following the successful development of insect cell lines, some attempts were made to develop cell culture methodologies for aquatic invertebrates, including sponges (Pomponi *et al.*, 1997), molluscs (Wen *et al.*, 1993; Domart-Coulon *et al.*, 1994), crustaceans (Chen *et al.*, 1986), and ascidians (Raftos *et al.*, 1990). Most of these species received attention, because their cells or tissues produce metabolites of possible pharmacological significance (Pomponi *et al.*, 1997), or because the host serves as a vector for pathogens (Gong *et al.*, 1997).

Fischer-Piette (1931) made the first attempt to establish tissue cultures for crustaceans, using simple saline solutions. Other studies were successful in establishing short-term crustacean cell cultures, but none were successful in establishing long-term cultures, and many were unable to subculture cells. As aquaculture began to develop and grow in the early 1980's, industry expansion was plagued by disease. The lack of crustacean cell lines for isolation and growth of crustacean viruses was a major setback for diagnosis and treatment. Most of the crustacean species in aquaculture come from the order Decapoda, of which Penaeidea (prawns), Astacidea (lobsters, crayfish), and Brachyura (true crabs) are representative orders. Penaeid species compose the largest group of crustaceans in aquaculture, with global production of approximately 3.16 million mt (FAO, 2006). Therefore, the penaeid species have been the primary focus of cell culture efforts during the past two decades. Since short-term tissue culture of *Penaeus monodon* was reported by Chen *et al.,* (1986), culturing efforts have been made on another eleven penaeid species (Hu, 1990; Rosenthal and Diamant, 1990; Ellender *et al.*, 1992; Luedeman and Lightner, 1992; Purushothaman *et al.*, 1998; Chen and Wang, 1999; Owens and Smith, 1999; Gao *et al.*, 2003; Maeda *et al.*, 2004). Primary cultures obtained from various penaeid organs have been reported with increasing frequency (Wang *et al.*, 2000; Mulford *et al.*,

2001; Uma *et al.*, 2002; Assavalapsakul *et al.*, 2003; Chen-Lei *et al.*, 2003; Gao *et al.*, 2003; Maeda *et al.*, 2003; Lang *et al.*, 2004; Maeda *et al.*, 2004; Jiang *et al.*, 2005). These studies represent the first steps towards the establishment of cell lines, and they provide useful information concerning suitable cell culture conditions for the survival and proliferation of various tissues (Toullec, 1999). To date, however, no valid and verified crustacean cell line has been established.

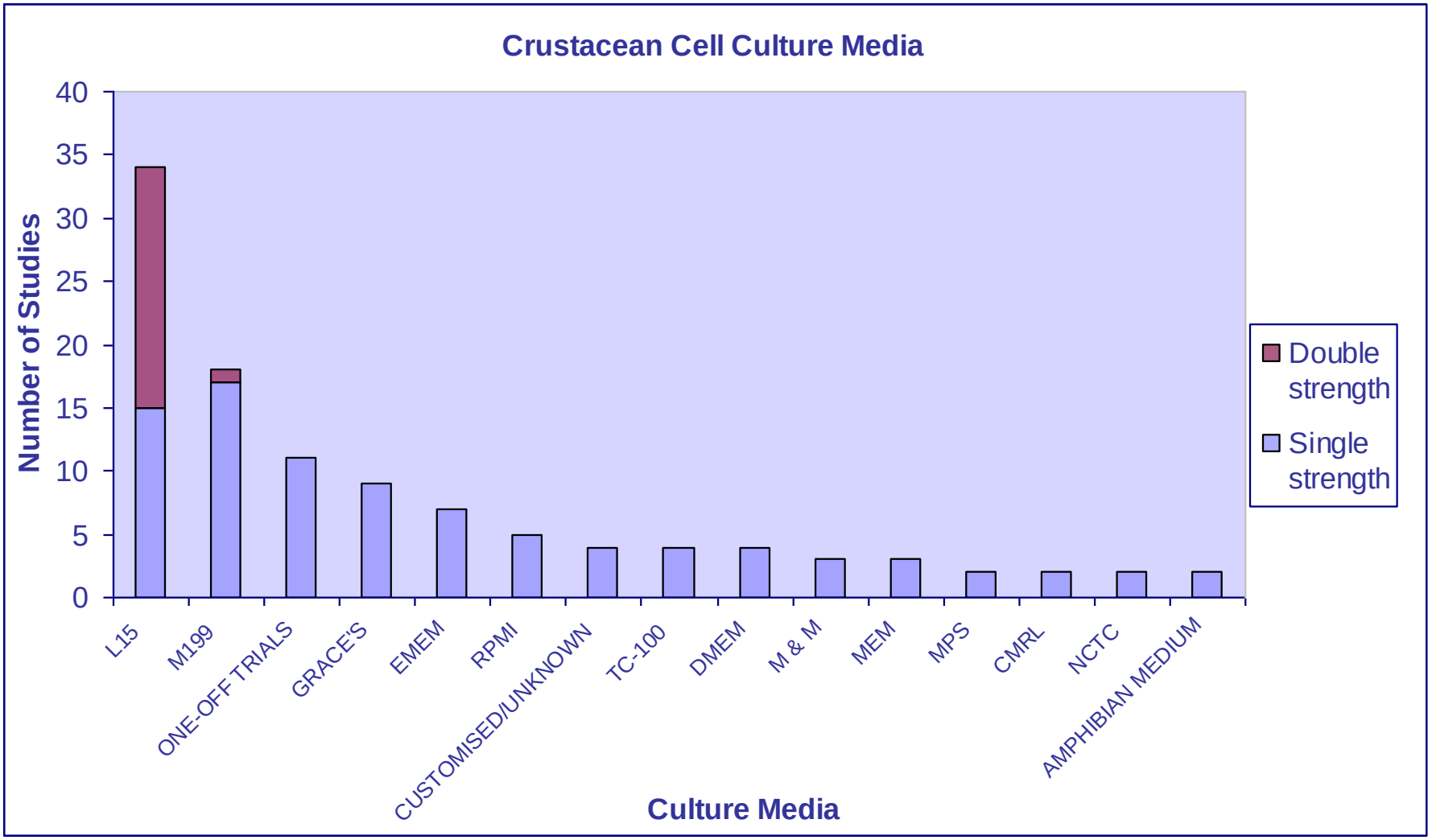
Development of a cell line provides many advantages. Unlike virology of aquatic vertebrates, crustacean virology is still in its infancy, and the major goal of cell line development has been to improve knowledge of disease-causing agents. *In vivo* models have limitations for studying disease, and isolation of pathogens *in vivo* is difficult. The production of standardised *in vitro* systems provides a means to study the effects and mechanisms of pathogen invasion and provides a medium for isolation of infectious pathogens. *In vitro* systems also allow the study of cellular biochemistry and can facilitate studies of ecotoxicology (Freshney, 2005). Once cell lines have been established, they reduce the need for animal experimentation, which precludes legal, moral and ethical problems, as cells can be continually cultivated without the need to sacrifice whole organisms. Cell cultures also allow the culture of a single virus, instead of the mixed populations of viruses that are worked with in current bioassay systems (Owens, 1997).

The purpose of this review is to summarise previous attempts at crustacean cell culture, and to compare this research with cell culturing efforts for other invertebrates and some vertebrates. Recent technological advances and novel approaches to cell culture are also discussed. Specific topics covered include crustacean cell culture media and their components, species and tissues used in cell culture studies, methods used to characterise cells in culture, viral infectivity, approaches to development of immortal crustacean cell lines, and problems related to crustacean cell physiology, cell sensitivity, and cellular senescence.

2.2 CELL CULTURE MEDIA

Most attempts at crustacean cell culture have focused on optimisation of the culture media. The culture medium is the most important element impacting on the survival of animal cells (Mothersill and Austin, 2000). The selection of culture medium is generally based on the requirements of the animal. With aquatic animals, this involves consideration of the external environment (marine or freshwater), the internal environment (specific requirements of the species), and whether carbon dioxide or any other buffering system will be used with the medium. There is virtually nothing known about the precise nutritional requirements of crustacean cells and, therefore, much of the medium selection process is guesswork (Mothersill and Austin, 2000).

Basic culture medium typically supplies essential nutrients for cellular growth, including salts, amino acids, and vitamins. Numerous studies have tested a variety of culture formulations (combinations of basal media and supplements) for their ability to support crustacean cell replication or maintenance. Since the development of more complex and defined media, many initial trials of crustacean cell culture utilised Grace's insect medium, due to its success in culturing other arthropod species (Luedeman and Lightner, 1992; Nadala *et al.*, 1993; Frerichs, 1996; Toullec *et al.*, 1996; Fraser and Hall, 1999). Twenty-five other media have also been tested with crustacean cells (Appendix 3); with Leibovitz's (L-15) and medium 199 (M199) the most commonly used (Figure 2.1).



M & M = Maramorosch & Mitsuhashi media

Figure 2.1. The variety of culture media used for crustacean cell culture studies. L-15 medium is the most common culture medium, and is used at single and double strength.

The media M199 and L-15 have consistently supported successful establishment of primary cultures from various tissues. These media are very different in their composition and the proportion of their components (Lang *et al.*, 2002a). Due to observed cellular growth in both media, it has been assumed that crustacean cells have a broad adaptability to media conditions (Lang *et al.*, 2002a). Because these commercial culture media were primarily developed for vertebrate cell cultures, it is likely that they do not contain all the necessary *de novo* factors for invertebrate cell replication. Therefore the basal medium may not be as important as specific additives or supplements, especially for prolonged survival.

Although many researchers have examined the benefits of different media and/or modifications, few studies have attempted to identify specific physiological needs of crustacean cells (Mothersill and Austin, 2000). The method often employed by researchers to determine the physiological requirements of crustacean cells involves qualitative observations by light microscopy: either cells replicate or do not. This has been the basis for designing and modifying crustacean cell culture media. No previous studies have approached crustacean cell culture by analysing cell metabolism, i.e. by examining metabolic pathways and analysing the role of each nutrient. To date, only two studies focused on biochemical analysis of crustacean haemolymph, and both found that cultured cells performed better (established monolayers or delayed onset of cellular senescence) when the culture medium was optimised (Najafabadi et al., 1992; Shimizu et al., 2001). However, even with the optimised media, there was no evidence of sustained DNA or protein synthesis.

Regardless of the choice of basal medium, the culture medium must be adjusted to the physico-chemical requirements of the crustacean cells, depending on whether the species is freshwater or marine. The main factors that must be considered are osmolality, pH, and ionic strength. The osmotic pressure should be adjusted to 400-500 mOsm/kg (of water) for freshwater animals, and to 800-1000 mOsm/kg for marine species (Mothersill and Austin, 2000). In adjusting osmolality, ionic strength must also be taken into account, because the use of sodium chloride alone may result in an excessive

concentration of sodium (Mothersill and Austin, 2000). Some salts appear to be more important than others, particularly calcium, which has been described as the most important ion for the effectiveness of saline solutions in sustaining viability of crustacean cells (Wheatly, 1999).

The culture media should be relatively alkaline (pH 7.2-8.0), and non-CO_2 dependent biological buffers, such as HEPES, appear to be preferable to bicarbonate buffer (Mothersill and Austin, 2000). However, in mammalian cell cultures, HEPES has induced vacuolisation of cultured cells in the absence or at low concentrations of serum (Bowers and Dahm, 1993).

It has recently been recognised that the medium-change regime may be more important than the selection of medium (Mothersill and Austin, 2000). Once the cells have been added to the medium, the culture develops a micro-environment that is optimal for growth and function of the cells. Too frequent medium changes can cause the loss of this micro-environment and destroy the developing cells. Conversely, medium changes are essential to ensure cells receive their specific nutritional requirements. Therefore, half medium changes may better sustain the microenvironment and, at the same time, replace exhausted nutrients (Mothersill and Austin, 2000).

Any medium chosen for cell culture should be refined to incorporate known requirements of the animal. Overall, cell culture media should be made up of a basal medium that includes essential amino acids and vitamins, have ionic strength and pH adjusted to the animal's physiology, and contain species-specific nutritional supplements.

2.3 CULTURE MEDIA COMPOSITION

To stimulate cultured crustacean cells to carry on cell division, culture media have been supplemented with various nutrients, tissue extracts, and growth factors. Selection of these supplements has been based more on trial and error than on determination of essential cellular growth factors.

The salt, amino acid, and lipid composition of haemolymph has been reported for two species of penaeids (Najafabadi *et al.*, 1992; Shimizu *et al.*, 2001), and the results demonstrate the variation in haemolymph composition within the genus *Penaeus*. Seasonal influences and varying water salinity had profound effects on haemolymph composition (Najafabadi *et al.*, 1992). Other haemolymph metabolites varied from sample to sample, suggesting differences may have been caused by variations in diet and stress (Najafabadi *et al.*, 1992). Therefore, it is not accurate to compare species from different locations with different environmental conditions and different feeding regimes, and attempt to formulate a generic medium to enable cell replication. Nevertheless, many culture media supplements, including proteins, lipids, carbohydrates, vitamins, amino acids and growth factors, have been tested by trial and error, and have assumed that a growing and, in some cases, dividing cell, has had its nutritional requirements satisfied.

2.3.1 Foetal Bovine Serum

Foetal bovine serum (FBS) is a common supplement to culture media. FBS is a complex supplement that acts as a source of minerals, lipids, and hormones, and contains growth and adhesion factors to promote cell attachment and proliferation (Freshney, 2005). The results of several studies indicated that FBS is essential for cell replication, although it is likely that does not provide all of the specific hormones and growth factors needed by crustacean cells (Mothersill and Austin, 2000; Mulford *et al.*, 2001; Crane and Williams, 2002). Concentrations of FBS in culture media typically range between 5 and 20%, adjusted according to the tissue being cultured. The lipids provided by FBS have been found to be poor in quality for the requirements of crustacean cells (Mothersill and Austin, 2000). There is a low concentration of cholesterol and an imbalance between fatty acids and phospholipids. Extra supplementation of the culture medium with these lipids may be necessary for culturing crustacean cells (Fox *et al.*, 1994; Mothersill and Austin, 2000). Mammalian serum may also lack hormones and growth factors needed by crustacean cells, so additional supplementation of these factors may be necessary to stimulate cell proliferation.

There is a current trend to replace FBS with defined serum-free supplements, due to the inconsistent quality and increasing cost of FBS. Yeastolate, lactalbumin hydrolysate, peptones, and tryptose phosphate broth have been tested as replacements for bovine serum (Mothersill and Austin, 2000; Freshney, 2005). Yeastolate, an enzyme-hydrolysed yeast extract composed of a mixture of amino acids, peptides, water soluble vitamins, and carbohydrates, has been used as additive for crustacean cell culture (Maeda *et al.*, 2003) and insect cell culture (Mendonca *et al.*, 1999; Leite *et al.*, 2003). It is often used in combination with lactalbumin hydrolysate, which reduces the cell requirements for serum by replacing required polypeptides. Yeastolate is also a source of peptide hormones and has been found to enhance cell replication and viability in crustacean cell cultures (Itami *et al.*, 1999; Lang *et al.*, 2002a; Lang *et al.*, 2002b; Assavalapsakul *et al.*, 2003; Maeda *et al.*, 2003). It would be of interest to determine whether serum replacements developed for mammalian cell culture, containing defined nutrients and growth factors, can enhance or replace FBS in crustacean cultures.

Another alternative to supplementation with FBS is the use of isogenic or allogenic muscle extracts or plasma from haemolymph. Many studies of crustacean cell culture have combined these additives with sera and achieved varying results (Chen *et al.*, 1986; Chen *et al.*, 1988; Rosenthal and Diamant, 1990; Nadala *et al.*, 1993; Lu *et al.*, 1995b; Tapay *et al.*, 1995; Tong and Miao, 1996; Chen and Wang, 1999; Fraser and Hall, 1999; Itami *et al.*, 1999; Kasornchandra *et al.*, 1999; Owens and Smith, 1999; Mulford *et al.*, 2001).

2.3.2 Proteins

One of the main reasons for adding serum to culture media is to provide proteins necessary for cell survival and growth. Although proteins are a major component of serum, the functions of many proteins *in vitro* remain unclear (Freshney, 2005). It may be that relatively few proteins are required, other than as carriers for minerals, fatty acids, and hormones (Mothersill and Austin, 2000).

The total protein concentration in crustacean haemolymph is highly variable depending on species, nutritional status, and moulting stage (Najafabadi *et al.*, 1992; Mothersill and Austin, 2000). Najafabadi *et al.,* (1992) found that double strength L-15, supplemented with lobster haemolymph and 10% serum, had a protein concentration one third of that measured in *P. aztecus*.

A number of different circulating proteins and proteases have been isolated and characterised in crustaceans. The release of some of these proteins from damaged or dying cells can lead to degradation of tissues and cells. Proteases, for example, play roles in homeostasis, such as regulation of endogenous proteases, haemolymph coagulation, and antimicrobial defence (Merriam-Webster, 2003). Inhibition of proteases may be important to tissues rich in these enzymes, such as the hepatopancreas (Mothersill and Austin, 2000). The protease inhibitor phenyl methyl sulfonyl fluoride has been used to prevent autolysis of hepatopancreas cells by digestive enzymes released from damaged cells (Toullec *et al.*, 1992). Other protease-inhibiting mixtures have been developed for cell culture to prevent proteolytic degradation by secreted proteins, but their effectiveness has not been tested with crustacean cells.

2.3.3 Lipids

Lipid requirements vary among different cell types and animal species. Lipid metabolism in crustacea, are controlled by the adipokinetic/hyperglycaemia hormones (Mothersill and Austin, 2000). Ingested lipids are enzymatically cleaved into diglycerides and monoglycerides in the gut lumen, absorbed, converted to phospholipids, and stored in the hepatopancreas (Lee and Puppione, 1978). Unlike vertebrates, which use fatty acids as the principle transport lipids, the principal circulating lipids in crustacea are phospholipids (Mothersill and Austin, 2000). Phospholipids synthesised by the hepatopancreas are released to the haemolymph, where they are available to other cells and tissues for structural and energetic processes. Chang and O'Connor (1983) found that lipogenesis from carbohydrate catabolism is probably a deficient metabolic route in crustaceans. Therefore, lipids from vertebrate sera used as supplements in culture media may not be adequate to support crustacean cell replication. For example, levels of phospholipids in

prawn haemolymph (350-540 mg/L) are much higher than culture medium supplemented with 10% FBS (Najafabadi *et al.*, 1992).

Crustaceans are incapable of *de novo* synthesis of sterols and require a dietary source of cholesterol for growth, development, and survival (Fox *et al.*, 1994). The recommended level of cholesterol in crustacean diets varies from 0.4 to 1.4%, depending on species, age, and physiological condition (Teshima, 1997). Each species has an optimal level; with low cholesterol levels inhibiting growth and development, and high levels retarding growth (Teshima, 1997). Hernandez *et al.,* (2004) found that *Cherax quadricarinatus* fed a 0.5% cholesterol diet had significantly higher weight gain than crayfish fed lower or higher levels of cholesterol. Kasornchandra *et al.,* (1999) found supplementation with 0.01% cholesterol markedly enhanced cell replication of penaeid cells. Overall, it is important that animals are supported with diets containing necessary fatty acids and cholesterol prior to tissue extraction for the establishment of cell cultures, and that the cell cultures are supplemented with these factors to enable cell survival.

2.3.4 Carbohydrates

Like most nutrients, glucose levels in crustacean haemolymph vary widely depending on the species, diet, season, and moulting stage. Glucose supplements are important for insect cell cultures (Hink, 1976), and is therefore included in most culture media as an energy source. It is metabolised principally by glycolysis to form pyruvate, which may be converted to lactate or to acetoactetate and enter the citric acid cycle to form CO_2 (Freshney, 2005). The accumulation of lactic acid in the medium suggests that the citric acid cycle many not function as *in vivo*. Carbon may be derived from glutamine, rather than glucose, and this may explain the high requirement of some cultured cells for glutamine (Freshney, 2005).

Many studies have found supplementation with glucose during the first 24 h of culture leads to rapid cell attachment and migration from explants (Hsu *et al.*, 1995; Kasornchandra *et al.*, 1999; Mulford *et al.*, 2001; Crane and Williams, 2002; Assavalapsakul *et al.*, 2003; Maeda *et al.*, 2003). Without glucose, the

number of cells that attach to culture vessels is considerably reduced. Enhanced cell migration has also been observed by continual addition of one g/L glucose (Hsu *et al.*, 1995; Mulford *et al.*, 2001; Maeda *et al.*, 2003). The need for supplemented glucose depends on the culture medium. L-15 is one of few culture media that contains galactose rather than glucose. Therefore, most studies that found the addition of glucose beneficial used L-15 as the basic culture medium (Hsu *et al.*, 1995; Kasornchandra *et al.*, 1999; Crane and Williams, 2002; Assavalapsakul *et al.*, 2003; Maeda *et al.*, 2003). Other carbohydrate supplements, such as sucrose and trehalose, have not been as successful as additives, resulting in a cell attachment rate of only 50%, compared to 80% when cultures are supplemented with glucose (Hsu *et al.*, 1995).

2.3.5 Vitamins

Vitamin requirements of crustaceans have yet to be completely defined (Harlioglu and Barim, 2004). Results reported in the literature suggest that invertebrates have vitamin requirements similar to those of vertebrates, and both are unable to synthesise carotenes. Thiamine, riboflavin, nicotinamide, pyridoxine, pantothenic acid, biotin, and folic acid are regarded as essential vitamins for animals. Biotin and riboflavin, considered essential vitamins that aid in carboxylation reactions important for cell energy production, are lacking in L-15 medium (Luedeman and Lightner, 1992). Selenium is also lacking in most defined cell culture media; however, the effect of supplementation in *P. monodon* lymphoid organ culture was not commented on (Hsu, 1995). Keating and Dagbusan (1984) found that cell populations from water fleas could not be maintained without selenium supplementation at 0.1 ppb. In mammalian cell cultures, selenium deficiencies are managed by adding vitamin E, which lessens the absolute requirement for selenium, but does not wholly substitute for it (Freshney, 2005).

Few publications have reported on vitamin supplementation *in vitro*. However, vitamin deficiencies in dietary feeds have been reported. Kumar *et al.,* (2004) found that carotenoid deficiency in the freshwater prawn *Macrobrachium rosenbergii* caused an increase in appendage deformity syndrome (ADS),

resulting in 100% mortality after 18 days. However, supplementation with carotenoids allowed full recovery from ADS, and permitted regeneration of appendages. Carotenoids, which include beta-carotene, alpha-carotene and beta-cryptoxanthin (Merriam-Webster, 2003), come primarily from fruits and vegetables, and provide the sole source of vitamin A in the diet. Crustaceans used for cell culture may require diets containing carotenoids or vegetable matter to ensure that vitamin A requirements are met prior to initiation of cells into culture. Vitamin A may also be a required constituent of the culture medium.

The dietary requirement for vitamin E has been reported to be 20-100 mg/kg for fish and crustaceans (Harlioglu and Barim, 2004). Higher levels of vitamin E can cause hypervitaminosis, which is evident as retardation in growth. A major function of vitamin E is to prevent peroxidation of polyunsaturated fatty acids in the phospholipid and cholesterol of cellular and subcellular membranes (Harlioglu and Barim, 2004). Vitamin E added at 100 mg/kg increased pleopodal egg number and survival of juveniles of the crayfish, *Astacus leptodactylus* (Harlioglu and Barim, 2004). Addition of vitamin E, along with vitamins A and D, significantly improved growth and survival of *P. vannamei* (He *et al.*, 1993). As most vertebrate-defined culture media probably do not provide all vitamins and minerals at optimal levels for cultured crustacean cells, experimentation with vitamin supplementation *in vitro* might be necessary to identify specific cellular requirements.

2.3.6 Amino Acids

The essential amino acids, i.e. those not synthesised within an organism, are required by cultured cells and should be included in any basic basal media. The individual requirements for amino acids vary from one cell to another, depending on cellular function (Freshney, 2005). Other, non-essential amino acids are often added to media, in case cells have lost their capacity to make them. The concentration of amino acids usually limits the maximum cell concentration attainable in cultures, and the balance may influence cell survival and growth rate (Mothersill and Austin, 2000). A list of the non-essential and essential amino acids of crustacean is listed in Table 2.1.

Table 2.1. The non-essential amino acids that crustaceans are able to synthesise and essential amino acids that must be supplied in the diet or culture medium.

NON ESSENTIAL AMINO ACIDS	ESSENTIAL AMINO ACIDS
Glutamine	Lysine
Proline	Arginine
Alanine	Valine
Glycine	Leucine
Asparagine	Threonine
Tyrosine	Histidine
Serine	Isoleucine
Glutamic acid	Phenylalanine
Aspartic acid	Methionine
Cysteine	Tryptophan

Source: (Claybrook, 1983)

2.3.6.1 Proline

Proline acts as an osmolyte in a wide variety of marine invertebrates (Yancey *et al.*, 1982), and is an energy source for insect flight muscles (Candy *et al.*, 1997). Proline is one of the major free amino acids in the haemolymph of some prawn species (Fang *et al.*, 1992; Shimizu *et al.*, 2001). Shimizu *et al.,* (2001) found that proline was 20-100 times lower in L-15 and M199, the two most frequently used culture media, than in the haemolymph *of P. stylirostris*. When proline was added to the medium, monolayers of cells were achieved, although there was no evidence of sustained DNA or protein synthesis. Mulford *et al.,* (2001) also found the addition of proline to be beneficial to haematopoeitic tissue culture in the Dublin Bay prawn, *Nephrops norvegicus*.

2.3.6.2 Glutamine

Glutamine is required by most cells, and its addition to the culture medium has been found to greatly enhance cell survival (Freshney, 2005). Glutamine is

used by cultured cells as a source of energy and carbon (Butler and Christie, 1994). Energy metabolism is a requirement of the cell culture system for cellular growth. Therefore, the citric acid cycle must remain active, and it has become apparent that amino acids, particularly glutamine, can be utilised as a carbon source by oxidation to glutamate (Freshney, 2005). The deamination of the glutamine tends to produce ammonia, which is toxic to aquatic cells and can affect protein glycosylation, thereby limiting cell growth. However, dipeptides appear to minimise the production of ammonia and have the additional advantage of being more stable in the medium (Freshney, 2005). Thus, if glutamine is to be utilised as a supplement in crustacean cell cultures, it may be more advantageous to use glutamylalanine or glutamyl-glycine.

2.3.7 Betaine

Betaine was first identified in sugar beets and was later shown to be present in many plants and in animals. As animals are unable to synthesise betaine, it must be present in the environment or food source. Betaine is a highly water soluble compound that functions primarily as an osmotic agent (Petronini *et al.*, 1993). Metabolised betaine is also a transient source of methyl (CH_3) groups, which are required for methylation reactions. For example, methyl groups from betaine may be used to convert homocysteine to methionine, an essential amino acid (Zeisel, 2008).

Ronsch *et al.,* (2003) investigated the impact of osmotic stress on lysine production in a bacterium and found that, under severe osmotic stress, proline was the prominent lysine-compatible solute in growing cells. Uptake of betaine, if available in the cell culture medium, reduced the concentration of proline from 750 to 300 mM, indicating that uptake of compatible solutes was preferred to synthesis. Furthermore, betaine was shown to have a higher efficiency to counteract osmotic stress than proline.

Few studies have examined effects of betaine on crustaceans. *Penaeus monodon* fed betaine exhibited reduced mortality when exposed to viral pathogens in the face of varying temperatures (Owens and Liessmann, 2004). Only two studies of crustacean cell culture have included betaine in the cell

culture medium (Ellender *et al.*, 1992; Najafabadi *et al.*, 1992). Neither study indicated the reason for its addition, or whether or not betaine was beneficial. Nevertheless, an osmotic agent capable of osmoprotection *in vitro* may be worth investigating.

2.3.8 Cytokines, Growth Factors, and Hormones

Cytokines are members of a protein family that are released by cells and have specific effects on cell behaviour, cellular interaction and communication (Merriam-Webster, 2003). Cytokines control cell growth and differentiation, and regulate immune and inflammatory responses. Although they are similar to hormones, cytokines can be released from a variety of cell types, rather than by specific cells, as is true for hormones. Cytokines that function as growth factors regulate several processes. Growth factors are essential to the normal cell cycle and thus, are vital elements in all animals. Growth factors may act synergistically or additively with each other or with other hormones and activity of some growth factors is dependent on the activity of a second growth factor (Phillips and Cristofalo, 1988). The results of four types of growth factors that have been experimented with in crustacean cell culture have been reported. These include insulin growth factors (IGF-I and IGF-II), epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), and transforming growth factor beta (TGF-β). Two other cytokines, interlukin-2 and a mouse nerve growth factor, have also been researched, however their ability to enhance cell growth *in vitro* was not commented on (Nadala et al., 1993).

EGF, like all growth factors, binds to specific high-affinity, low-capacity receptors on the surface of responsive cells. Tyrosine kinase is intrinsic to the EGF receptor, and tyrosine kinase activity is stimulated by EGF binding. In cell cultures, EGF has proliferative effects on cells of both epidermal and epithelial origin, particularly keratinocytes and fibroblasts (Freshney, 2005). In crustacean cell culture experiments, EGF stimulated or inhibited cell proliferation, depending on the cell type in culture. Lymphoid organ cultures of *P. monodon* treated with EGF at 3 ng/ml had higher attachment rates, but died earlier than control cells *(*Hsu *et al.,* 1995). EGF at 10 ng/ml also increased the attachment of *P. monodon* ovary cells, but was detrimental to overall cell

survival (Fraser and Hall, 1999). At 20 ng/ml, EGF markedly enhanced *P. stylirostris* and *P. vannamei* lymphoid organ cell monolayers, but long term cell viable was not reported (Nadala *et al*., 1993). In contrast, Cancre (1995) successfully cultured hepatopancreatic cells of *P. serratus* using EGF at 60 ng/ml, and concluded that EGF stimulated protein synthesis. However, Mulford *et al.,* (2001) found EGF had no appreciable effect on haematopoietic cells in *N. norvegicus*. Other studies that have utilised this growth factor have not commented on the effects of its addition to culture media (Lu *et al.*, 1995b; Tapay *et al.*, 1995). Further investigation is necessary to establish the effect of EGF on crustacean cells under various culture conditions.

Previous studies using mammal cells showed that basic fibroblast growth factor, bFGF, can bind to heparin-like molecules in the extracellular matrices of endothelial cells (Presta *et al.*, 1989), thereby stimulating cell replication and differentiation (Ingber and Folkman, 1989). Moscatelli and Quarto (1989) demonstrated that high bFGF concentrations reduced the number of bFGF receptors and induced cell transformation in mouse cells. Four studies examined effects of bFGF on crustacean cell cultures. Hsu *et al.,* (1995) found that lymphoid cells of *P. monodon* treated with bFGF at 20 ng/ml grew quickly and developed an extracellular matrix for cell attachment and growth. These cells also grew in suspension and could be subcultured more than 80 times. Fan and Wang (2002) showed that bFGF at 20ng/ml in combination with IGF-II at 80ng/ml elicited cell proliferation in embryonic cells of *P. chinensis*. In contrast, Mulford (2001) found that bFGF at 10-50 ng/ml did not produce any appreciable changes in cultured haematopoietic cells. Similarly, Fraser *et al.,* (1999) concluded that bFGF at 20ng/ml had no effect on ovary cells of *P. monodon* in culture, and Crane and Williams (2002) found that addition of bFGF (20ng/ml) did not promote cell growth and division in a number of tissues.

IGF-I and IGF-II are similar to insulin in structure and function. Insulin is a peptide hormone that enhances the transportation and utilisation of sugar, as well as the absorption of amino acids by cells (Freshney, 2005). Insulin also stimulates DNA synthesis in some cells and acts as a growth factor (Hsu *et al.*, 1995). IGF-I can stimulate cell proliferation after binding to cell receptors;

however, *P. monodon* lymphoid cells treated with IGF-I at 25 ng/ml became contaminated with yeast and had to be discarded (Hsu *et al.*, 1995). IGF-I at 5-20 ng/ml had no observable stimulatory effect on haematopoietic cells in the Dublin prawn (Mulford *et al*., 2001)**.**

IGF-II is almost exclusively expressed in embryonic tissue, and is required for early development (Pimentel, 1994). IGF-II has only been used in one study of crustacean cell culture. In combination with bFGF, IGF-II enabled embryonic cells of *P. chinensis* to grow rapidly and form confluent monolayers in three days (Fan and Wang, 2002). The cells could also be subcultured, providing they were continuously exposed to both growth factors. In other invertebrate cell cultures, Hatt *et al.,* (2001) investigated the effects of IGF-I and IGF-II on a lepidopteran cell line, and found these supplements enabled the progression of the cell cycle, as evidenced by 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis, but did not stimulate mitotsis.

The transforming growth factor beta (TGF-β) has only been utilised in crustacean cell culture on one occasion. Hsu *et al.,* (1995) found that lymphoid cells from *P. monodon* treated with TGF-β died within three days. Baker and Reddy (1998) found that TGF-β along with another cytokine, tumor necrosis factor-alpha (TNF-α), were potent and rapid inhibitors of proliferation, and induced growth arrest and apoptosis in vertebrate cells. A study of cultured cells from the insect, *Lymantria dispar,* showed that TGF-β1, along with platelet-derived growth factor, PDGF-AB, partially inhibited the programmed cell death induced by a classical apoptosis inducer of mammalian cells (Ottavianai *et al.*, 2004). The same study demonstrated that PDGF-AB and TGF-β1 are involved in the release of biogenic amines, which can be toxic to cells. PDGF-AB inhibited, and TGF-β1 stimulated amine release. Thus, PDGF-AB may be suitable as a growth stimulant for cultured crustacean cells.

Crustacean hormones regulate many different aspects of physiology, including larval development, growth, moulting behaviour, reproduction, and internal homeostasis (Mothersill and Austin, 2000). Supplementation with vertebrate serum, usually FBS, provides many of the hormones and growth factors

necessary for cultured cells. However, there is no evidence that FBS provides all the hormones and growth factors required by crustacean cells (Mothersill and Austin, 2000; Mulford *et al.*, 2001; Crane and Williams, 2002). Haemolymph and tissue extracts have been used as another source of hormones and growth factors (Chen *et al.*, 1986; Chen *et al.*, 1988; Rosenthal and Diamant, 1990; Nadala *et al.*, 1993; Lu *et al.*, 1995b; Tapay *et al.*, 1995; Tong and Miao, 1996; Chen and Wang, 1999; Fraser and Hall, 1999; Itami *et al.*, 1999; Kasornchandra *et al.*, 1999; Owens and Smith, 1999; Mulford *et al.*, 2001). None of these studies analysed the components of the tissue extract and or haemolymph additive, so the specific hormones or growth factors present were not known. Formulation of crustacean-defined media requires a hormonal profile, which would probably vary depending on the species and tissue used in culture.

2.4 CRUSTACEA USED IN CELL CULTURE TRIALS

Efforts to develop crustacean cell lines have recently intensified, with emphasis on the commercially important penaeid species (Figure 2.2). Penaeids succumb to many viral infections in aquaculture and serve as excellent models for pathological studies. The most popular species used in cell culture experiments is *P. monodon* largely due to its worldwide availability and its popularity in aquaculture. *Penaeus japonicus* has been used in eight reported cell culture trials, *P. stylirostris* in six, and *P. vannamei* in five. Other penaeid species have been used in only one or two cell culture trials. Only four crustacean species other than prawns have been investigated for cell culture: the sand crab, *Emerita asiatica*; the swimming crab, *Liocarcinus depurator*; North American lobster, *Homarus americanus*; and the crayfish, *Orconectes limosus.* Out of the 17 species that have undergone cell culturing efforts, only two species are of freshwater origin, and the rest are marine (Appendix 3).

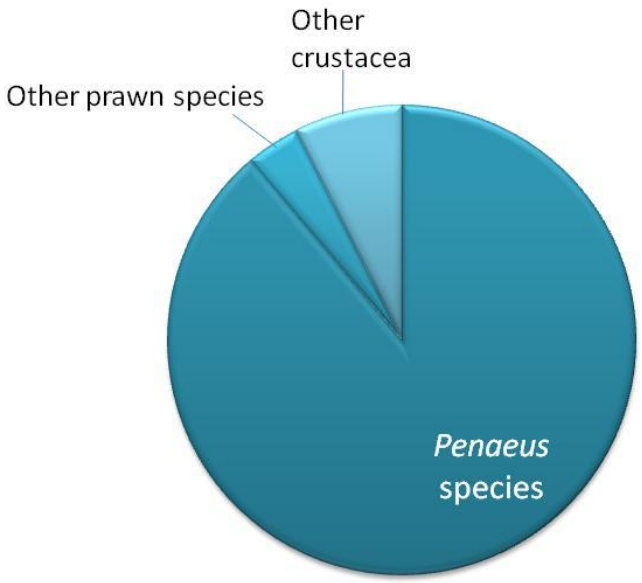


Figure 2.2. Crustacean species used in cell culture attempts.

2.5 TISSUES USED IN CRUSTACEAN CELL CULTURE

There is much disagreement regarding the best tissue for crustacean cell culture, which might be attributed to the different media and species being tested (Tong and Miao, 1996). Nevertheless, a few conclusions can be drawn on the basis of published results.

First, the age of the animal used for cell culture is likely to have a significant impact on cell proliferation; the younger the animal used as a tissue source, the more successful cell culture initiation is (Tong and Miao, 1996). Embryonic tissues provide undifferentiated and rapidly multiplying cells, which tend to adapt to *in vitro* growth more readily than the specialised cells of mature shrimp (Fan and Wang, 2002). Larval and embryonic cells in culture, furthermore, are less vulnerable than cells from adult animals to contamination from bacteria, yeast, and mould when attached to a substrate (Frank *et al.*, 1994; Rinkevich, 1999) and have sometimes been found to survive culture conditions longer (Rinkevich, 1999; Fan and Wang, 2002).

The second conclusion from previous studies of crustacean cell culture is that tissues selected for culture should contain cells that are mitotically active. Embryonic tissues satisfy this criterion, as these tissues contain many undifferentiated and rapidly multiplying cells (Frerichs, 1996). Ovary tissue has also been a popular choice, as it is easy to locate, and promising for cell line establishment because of the numerous mitotically active germ stem cells (Luedeman and Lightner, 1992). Owens and Smith (1999) suggested that germinal tissues, such as haematopoietic, gonadal, and gut proliferative zones (e.g. distal hepatopancreatic cells and midgut caecae), subcutical areas at premoult, and embryonic tissues may provide the best source of proliferating cells. Neumann *et al.,* (2000) may have created immortal cell cultures using neuronal and haematopoietic stem cells of the crayfish, *Orconectes limosus,* yet this lacks verification. No manipulation was carried out to transform cells, other than the selection and cloning of potential stem cells. These cells showed features of transformation, such as aneuploidy, anchorage independence, loss of contact inhibition, and low serum requirement. However, this cell line has not been verified or investigated for its ability to support viral growth. One of the main goals for the development of crustacean cell culture is disease diagnosis. Therefore, the potential cell line must be able to support viral growth *in vitro*. This is the reason why the lymphoid organ has been frequently selected as a tissue source. The lymphoid organ is the target for many infectious agents, so cells of the lymphoid organ are suitable for studying the mechanism of pathogen infection to host. To date, the longest survival time for primary culture of lymphoid organ cells is 43 days (Kasornchandra *et al.*, 1999). Cells of the hepatopancreas have also been used to investigate the effect of disease and were maintained for 12 weeks (Uma *et al.*, 2002). Many other tissues have been extracted from crustaceans in attempts to develop proliferating cell populations (Table 2.2).

Table 2.2. Crustacean tissues used in cell culture attempts.

TISSUE	NUMBER OF TRIALS
Lymphoid organ	19
Ovary	18
Hepatopancreas	12
Heart	9
Muscle	8
Embryo/Larvae	6
Haemocytes	6
Haematopoietic	5
Gill	5
Nerve	4
Neuronal/eyestalk	6
Eye	3
Epithelium	3
Testis	2
Antennae	1
Gut	1
Regeneration buds	1

The method used to obtain cells from tissues influences the survival of the cells in culture. Two different methods are used: explants, in which cells migrate from the tissues, and dissociation (Toullec, 1999). In explants, tissues are removed using sterile techniques and placed in vessels containing the selected culture medium, based on the idea that cell aggregates have better ability than single cells to adhere to the substrate (Yavin and Yavin, 1974). This method has the potential to provide a large number of cell types and reduce stress to the cells; however, it is best adapted to loose tissues (Toullec, 1999).

Cell dissociation involves seeding culture flasks with tissues disrupted by either mechanical or enzymatic means. Mechanical dissociation can be achieved with magnetic stirring or by repeated aspiration using a pipette or syringe (Toullec, 1999). This technique is also suitable for loose tissues and can provide a large number of cells, but seems to reduce the ability of cells to attach to the substrate (Yavin and Yavin, 1974). Fragile cell types are often broken and ruptured during mechanical treatment, releasing proteases into the medium which leads to cell digestion (Freshney, 2005).

Enzymatic cell dissociation uses enzymes such as trypsin, pronase, collagenase, or enzyme mixes, such as collagenase/dispase. All enzymes are

not suitable for every tissue, and care must be taken to choose an appropriate enzyme. Trypsin, pronase, and collagenase seem to be too potent for crustacean tissues (Rosenthal and Diamant, 1990; Mothersill and Austin, 2000). Enzymatic dissociation is necessary for compact tissues, but can also be useful for loose tissues. This process is less drastic than mechanical dissociation and causes less cellular rupture. However, enzymatic treatment can weaken the cell membranes and decrease their ability to attach to the substrate. Cell cultures developed using enzymatic cell dissociation have been found to be capable of survival similar to cultures initiated by mechanical cell disruption. However, cell multiplication in enzyme dissociated tissues is low, and cell proliferation is limited upon sub-culture (Mothersill and Austin, 2000).

2.6 CHARACTERISATION OF CULTURED CELLS

2.6.1 Cell Identity

The characterisation of cells in a cell culture involves confirming that the cells are authentic to the species and tissue of origin. Transformed cell lines can lose characteristics of the primary cultures from which they are derived (Crane, 1999). Therefore, identification of the cell types in culture is necessary, and selection of cell types that produce desired effects (e.g. virus susceptibility) is also important. To date, characterisation of crustacean cultures has been based mainly on determination of cellular morphology by light microscopy (Tong and Miao, 1996; Mulford *et al.*, 2001). However, a variety of other methods have been developed, including immunohistochemistry, DNA fingerprinting, and identification of genes through the polymerase chain reaction (PCR).

Immunohistochemistry is one of the best methods for characterising specific phenotypic antigen expression (Freshney, 2005). Polyclonal or monoclonal antibodies have been developed for many crustacean molecules, including hormones, such as hyperglycaemic hormone, moulting inhibiting hormones, vitellogenesis-inhibiting hormone, and red pigment-concentrating hormone; neuropeptides, such as GABA, acetylcholine, serotonin, and histamine; and haemocytes. Antibodies or antiserum to phylogenetically conserved markers can also be used to identify crustacean cells in culture, including components of

the cytoskeleton, such as cytokeratins, actin, and myosin; specific enzymes, such as cytochromes and ATPases; and other proteins, such as heat shock proteins (Mothersill and Austin, 2000; Mulford *et al.*, 2001). Results of previous studies demonstrates that some antibodies to mammalian epitopes cross-react with crustacean epitopes. DNA stained with the fluorescent marker, propidium, was used to differentiate hepatopancreas cells from chytrid parasites in cell cultures of the midgut of *P. japonicus* (Machii *et al.*, 1988). Antigenic characterisation was achieved with hepatopancreas cells of *N. norvegicus* (Lyons-Alcantara *et al.*, 1999) and a variety of tissues from the crustacean *Pandalus borealis* (Lyons-Alcantara *et al.*, 2002). Thus, some cellular proteins are highly conserved in evolution and can be used to characterise crustacean cells *in vitro*.

DNA fingerprinting at the population level has permitted differentiation among species and has been successfully used with crustaceans. DNA fingerprinting can also identify a cell by visualising the structure of the repetitive component of genomic DNA, which is compared to a library of multilocus DNA fingerprints. The unique fingerprints enable the identification of cross-contamination, cell line variation, instability of cell lines, and inconsistent quality in cell stocks. This technique has been demonstrated to be both reliable and valuable for the identification of insect cell lines (Trisyono *et al.*, 2000); however, it has not yet been applied to crustacean cell cultures.

Polymerase chain reaction (PCR) technologies can also be utilised to identify species-specific genes or DNA. DNA can easily be extracted from cells, and primers of highly conserved gene regions, either from crustaceans or species-specific markers can be used. These methods have been used to authenticate many vertebrate cell lines, including human, monkey, mouse, rats, and fish (Capasso *et al.*, 2003). PCR amplification of the mitochondrial 18S rRNA gene fragment has also been used to characterise insect cell lines (Kshirsagar *et al.*, 1997).

2.6.2 Cell Viability

Although a cell culture may appear healthy, it is essential to verify cell viability and the capacity of cells to divide, to confirm the existence of a growing culture (Mothersill and Austin, 2000). The most common methods of assessing cellular viability involve the exclusion or uptake of various dyes. Trypan blue, for example, is a dye that is excluded by viable cells, but enters dead cells and stains them blue. Cells are then counted microscopically, and the number of viable cells is estimated. Viable cells can also take up fluorescent dyes and be counted using a flow cytometer (Freshney, 2005). In cells with compromised membranes, the fluorescent dye reacts with free amines in the cell interior and on the cell surface, yielding intense staining. In viable cells, the dye reacts only with amines on the cell surface, resulting in less intense fluorescence. Differences in fluorescence intensity are preserved during fixation of the cells (Perfetto *et al.*, 2006). Unfortunately, these viability tests are rarely applied to cell cultures from aquatic invertebrates (Mothersill and Austin, 2000), because it is difficult to remove viable cells from culture vessels, and the cells are sensitive to trypsinisation.

Another method of assessing cell viability involves colourimetric assays. Tetrazolium salts, a group of organic salts that are reduced to a coloured formazan by metabolically active cells, can also be used to detect viable cells. This method allows numerous colourmetric samples to be measured directly in a microtiterplate, using an enzyme-linked immunosorbant assay (ELISA) plate reader, and has proved successful for measuring proliferation in cell cultures from insects (Hatt *et al.*, 2001), molluscs (Lebel *et al.*, 1996), and algae (Capasso *et al.*, 2003). However, this technique has not yet been applied to crustacean cell cultures.

2.6.3 Cell Proliferation

Cell replication is a crucial aspect of cell culture (Doyle and Bolton, 1994). Quantitation of cell proliferation is important in routine maintenance, as it provides a method for monitoring the consistency of the culture. Proliferation is particularly important in measuring cellular responses to a stimulus, such as an experimental additive or supplement. Moreover, some protozoans residing in

the tissues are able to proliferate in the culture media, forming outgrowths that have been misinterpreted as true animal cell cultures or cell lines (Pomponi *et al.*, 1997). Proliferating fungal cells can also mimic haemocytes in cell culture (Rosenthal and Diamant, 1990). Assessment of cell proliferation may be done by mitotic counting, uptake of BrdU, tritiated-thymidine labelling, image analysis, flow cytometry, and immunohistochemical detection of cell-cycle proteins (Mothersill and Austin, 2000). In most of crustacean cell cultures, however, only primary cultures have been obtained, and cell proliferation has not been accurately recorded.

Two DNA methods are used to determine cell proliferation: tritiated-thymidine and BrdU incorporation. Both allow the labelling of cells only in the process of DNA synthesis. A labelling index is calculated as the number of labelled cells as a fraction of non-labelled or total cells. The tritiated-thymidine method uses radioisotopes, which involves safety issues, and the resulting autoradiographs can be difficult to interpret (Davis, 1994).

A variation of the tritiated-thymidine method uses the thymidine analogue, BrdU, which can be applied in a non-radioactive form and detected immunocytochemically (Hume, 1990). Actively replicating cells are identified by an immunocytochemical assay for the detection of BrdU incorporated into cellular DNA, using a mouse monoclonal antibody (MAb) (Gratzner, 1982). BrdU incorporated into the nucleus is a very stable antigen, which gives strong and reliable signals. Immunocytochemical staining for BrdU is considered an accurate method for measuring cell proliferation (Mothersill and Austin, 2000; Mulford *et al.*, 2001; Crane and Williams, 2002; Maeda *et al.*, 2003), and commercial kits are available. Crane and Williams (2002) successfully used this proliferation measurement, with minor adjustments, for finfish cell lines, but were unable to apply it to prawn cell cultures, indicating that the prawn cells were not undergoing DNA synthesis at the time of incubation with BrdU. Mulford *et al.,* (2001) had similar problems applying the method to cultures of the haematopoietic tissue of the Dublin prawn, which lacked cellular proliferation. However, cell proliferation in primary cell cultures of prawn tissues was successfully measured in two studies. Shike *et al.,* (2000) reported that

maximal BrdU incorporation occurred 6-24 h post-inoculation in 10-15% of *P. stylirostris* lymphoid organ primary cultures. Maeda *et al.,* (2003) found that 34% of ovary tissue cells from *P. japonicus* incorporated BrdU 24 h post-inoculation. However, there is some disagreement that these methods are not true assays of cell proliferation, but only measure the amount of DNA synthesis in a culture (Mothersill and Austin, 2000).

2.7 VIRAL INFECTIVITY STUDIES

The importance of animal cell cultures for the study of viruses is well established, especially for vertebrates (Luedeman and Lightner, 1992). Development of cultured cell monolayers, particularly continuous cell lines, in the 1950s, greatly facilitated viral isolation, cultivation, quantification, and characterisation (Crane, 1999). The basic techniques established then are still in use today and play an important role in disease diagnosis. The ability to grow viruses in culture also provides a potentially limitless source of the virus, facilitating viral research. Several viral pathogens are known to have devastating effects on crustacean populations, and cell lines can be utilised as a rapid diagnostic tools for pathogen detection.

Due to multibillion dollar losses experienced in crustacean aquaculture due to viral diseases (Johnson *et al.*, 2008), there is continuing interest in the development of crustacean cell lines for disease investigation. The current lack of continuous crustacean cell lines suitable for the isolation and growth of viruses is a major setback for disease diagnosis and treatment. For the last three decades, crustacean viral infections have been investigated using *in vivo* experiments involving infectivity bioassays and histopathology (Maeda *et al.*, 2003). These methods are time-consuming and inadequate when screening animals for early or carrier-state infections.

Limited progress has been made in the development of cell cultures for expression of prawn viruses, and no commercial crustacean cell line is currently available, despite the widely acknowledged need. However, several investigators have demonstrated that primary cultures of prawn cells can

support *in vivo* growth of certain viruses. *In vitro* growth in primary cell cultures has been reported for monodon baculovirus (Chen and Kou, 1989), yellow head virus (YHV; Lu *et al.*, 1995b; Chen and Wang, 1999; Assavalapsakul *et al.*, 2003), whitespot syndrome virus (WSSV; Chen and Wang, 1999; Uma *et al.*, 2002; Maeda *et al.*, 2004; Jiravanichpaisal *et al*., 2006), penaeid rod-shaped baculovirus (PRBV; Itami *et al.*, 1999), and systemic ectodermal and mesodermal baculovirus (SEMBV; Kasornchandra *et al.*, 1999). Most studies that used primary cell culture for viral isolation utilised the lymphoid organ as the tissue source for cells (Chen and Wang, 1999; Wang *et al.*, 2000). The replication strategies of WSSV were also studied on ovary (Maeda *et al*., 2004) hepatopancreas (Uma *et al.*, 2002) and haematopoietic cells (Jiravanichpaisal *et al*., 2006). Four studies investigated the effects of YHV on primary lymphoid organ cell cultures, providing methodologies for studying YHV-host cell interactions. An *in vitro* assay of YHV infectivity has also been developed (Lu *et al.*, 1995b). A study using this assay demonstrated that the highest titres of YHV were found in the lymphoid organ, suggesting that this organ is the primary target tissue for the virus (Lu *et al.*, 1995b).

The typical cytopathic effect (CPE) of a virus in cell culture, include rounding of the cells, detachment and lysis from the culture flask, followed by the development of circular clear areas with depletion of cells (Figure 2.3). These studies of viral infectivity in crustacean primary cell cultures have built the foundation of an important tool for basic research on prawn viruses, and the study of their interaction with the host organism.

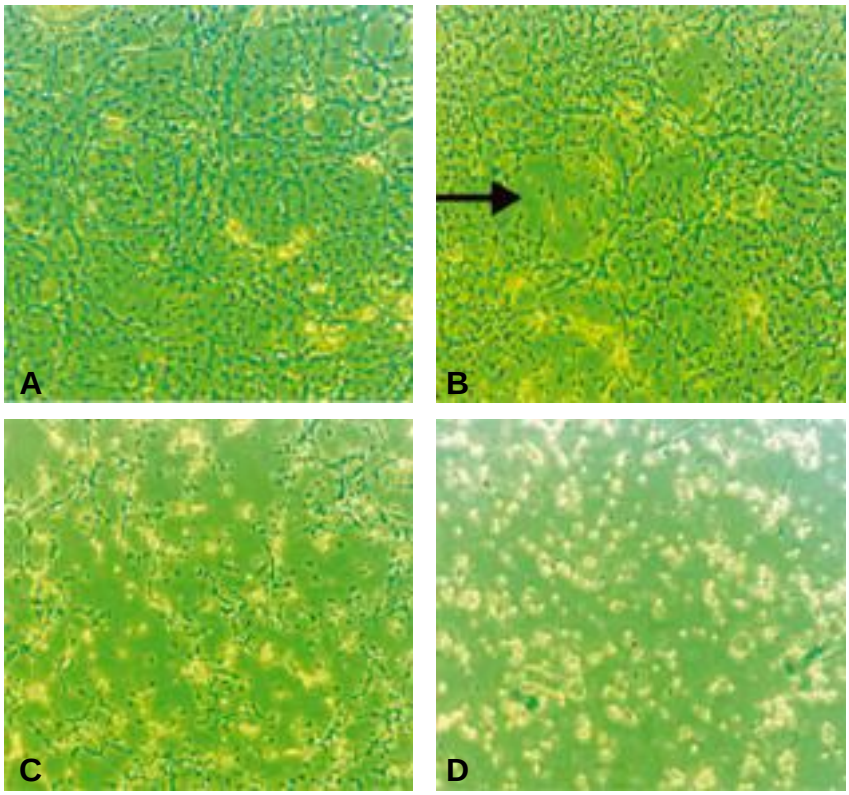


Figure 2.3. Cytopathic effects of yellow head virus in cell culture. (A) Confluent monolayer of cells before inoculation with virus. (B) Beginning of cytopathic effects at 3 days post-inoculation (arrow). (C) Five days post-inoculation, note large areas of clearance with cells beginning to detach. (D) Complete lysis at 7 days post-inoculation. Source: Assavalapsakul *et al.,* 2003.

At present, viral isolation in cell culture is the most sensitive and reliable technique for the detection of viral pathogens in fish (Crane, 1999). Viral isolation in cell culture is also used in the international trade of salmonid products, to certify stocks as disease-free and to act to control the spread of viruses (Crane, 1999.). Similar regulations may be required in the future for international trade of penaeid products, pending development of a penaeid cell line. The development of a penaeid cell line would also eliminate the need to sacrifice whole organisms and would provide a standardised system that is free of any other foreign entity.

2.8 DEVELOPMENT OF IMMORTAL CELL CULTURES

The majority of published research on crustacean cell culture is concerned with primary cell cultures (Crane, 1999). Primary cultured cells can only carry on limited cell division *in vitro*; the establishment of an immortal cell culture, or cell line, involves continuous cell division. The precise mechanism involved in the immortalisation of cells remains unknown. Several methods successfully transformed cultured mammalian cells, providing promising avenues for development of immortal crustacean cell cultures. Transformed cells have many advantages.

1) Establishment of primary cell cultures can be a tedious, labour-intensive and time-consuming technique. Immortal cell lines can be continuously passaged, with no need to return to whole animals as the source of cells.
2) Continuous cell lines are clonal and, therefore, relatively homogeneous with respect to genotype and phenotype.
3) Continuous cell lines can be mass cultured within a short period, which may be essential in the face of a serious and extensive disease outbreak (Crane, 1999).

To establish crustacean cell lines, a number of avenues should be explored, including identification of potential transformation techniques; examination of crustacean cell growth factors; investigation of transgenesis and mutagenesis of cells, cell hybridisation, suppression of cellular apoptosis; and a focus on secondary and cloned cells for homogenous cultures.

2.8.1 Transformation

Cell transformation can be either spontaneous or manipulated. Mutation based on physical, chemical, and molecular biological methods can increase the chance of cell transformation in comparison to spontaneous transformation. Transformation by mutational events has led to the development of numerous mammalian carcinogenic cell lines; however, it is likely that this transformation is a multi-step process and involves several genes (Crane, 1999).

A number of chemicals have been used to transform primary cell cultures into cell lines, including methylcholanthrene, azoxymethane, benzo(α)pyrene, and ethyl methanesulphonate (Davis, 1994; Freshney, 2005). Other chemicals are

effective at inducing point mutations, rather than immortalisation (Crane, 1999). Dose appears to be a critical factor. In a study where human cells were transformed with a chemical mutagen (4-nitroquinoline 1-oxide), immortalisation was achieved only with extensive, repeated treatments using low doses (Bai *et al.*, 1993). In general, prolonged use of relatively low concentrations of mutagenic agents that are more clastogenic in their mode of action may be more successful at inducing the changes required to effect transformation and allow selection of immortal genetic variants (Crane, 1999).

A number of viruses and viral genes have been used to immortalise cells in culture (Freshney, 2005). For example, the simian virus SV40, has been used to transform mouse, rat, bovine, rabbit, hamster, and human cells (MacDonald, 1990), and the large T antigen appears to be responsible (Brodsky and Pipas, 1998). SV40 was also used in an attempt to transform cells of *P. stylirostris* (Tapay *et al.*, 1995). However, in the case of human cells, many of the cell lines treated with SV40 are not truly immortal, but merely display a delay in the onset of senescence (Huschtscha and Holliday, 1983), and this may also be the case for potentially transformed cells of *P. stylirostris*.

Immortalisation of cells by viral genes usually occurs in two stages (Colgin and Reddel, 1999). In the first stage, there is a finite extension of proliferative potential, accompanied by telomere shortening. This stage ends in a period of culture crisis, when the culture stops expanding due to increased cell death from senescence. In the second stage, a small number of cells escape from crisis and acquire an apparently unlimited ability to proliferate, while telomere length is maintained (Colgin and Reddel, 1999). It is these cells that exhibit truly immortal characteristics. Escape from crisis may be associated with acquisition of telomerase activity or maintenance of telomeres via an alternative mechanism (see Section 2.9.4).

Other viruses and viral genes have been used to immortalise cells, including adenovirus (E1a), Epstein Barr virus, polyoma virus, and various leukaemia viruses. However, no virus other than SV40 has been trialled with crustacean cells. While cellular transformation with viral oncoproteins can extend cell

lifespan, it may fail to prevent telomere loss (Bodnar *et al.*, 1998). Therefore, telomere maintenance mechanisms may also need to be investigated, if crustacean cells are transformed by viral oncoproteins.

2.8.2 Transfection and Transgenesis

Another approach to developing cell lines is to introduce foreign genes, including viral vectors, into cells. The genes are cloned into plasmid vectors and introduced into primary cells by a variety of methods. DNA can be readily taken up by cells, if it is formed into a co-precipitate with calcium or strontium phosphate and Hepes buffer (Davis, 1994). The precipitate, which is believed to protect the DNA from degradation, is sedimented out and adsorbed onto the cell membrane. The DNA precipitate appears to be taken up by the cells through a calcium-dependent process (Davis, 1994).

Electroporation is another technique used for transgenesis of cells that are not suitable for co-precipitation. Cells are immersed in a DNA solution and subjected to an electrical field (Freshney, 2005). Pores form transiently in the cell membrane, through which the DNA enters. Cationic liposomes have been used successfully for both *in vitro* and *in vivo* gene transfer. However, some vertebrate cell types have proven very difficult to transfect using these liposome preparations. Histone or histone-like proteins have been demonstrated to be more effective mediators of transfection, with reports of increased transfection efficiency compared to liposome-based transfections (Esser, *et al*., 2000; Weng *et al*., 2004). Liua and Soderhall (2007) experimented with histone H2A as a transfection agent on haematopoietic cells in the freshwater crayfish *Pacifastacus leniusculus*, and reported a high transfection efficiency which was also non-cytotoxic to the crustacean cells. However liposome transfection is the only transgenesis method that has been attempted with crustacean cell culture (Tapay *et al.*, 1995). Another method of DNA delivery, micro-injection, has also proven to be very efficient. DNA was successfully delivered to *P. japonicus* embryos using microinjection, and this approach was more efficient than electroporation (Preston *et al.*, 2000).

A transfection reagent, jetPEI™, has also been successfully used to deliver genes to established mammalian cell lines, as well as to primary cells (Boussif *et al.*, 1995; Murphy *et al.*, 2001). DNA transfection by jetPEI™ is polyethylenimine-mediated: DNA is compacted into positively charged particles, capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. jetPEI™ possesses the unique property of acting as a "proton sponge," which buffers the endosomal pH and protects DNA from degradation. Continuous proton influx also induces endosomal osmotic swelling and rupture, which provides an escape mechanism for DNA particles to the cytoplasm (Derouazi *et al.*, 2004). Venzon *et al.,* (2004) compared the efficiency of microinjection, electroporation, and transfection (jetPEI™) for gene transfer into shrimp zygotes, and concluded that the transfection reagent was the most efficient method. The introduction of genes into crustacean cell cultures by transfection or other transgenesis methods merits further exploration.

2.8.3 Cell Hybridisation

Cell hybridisation is the process by which two dissimilar cells fuse together, forming a binucleated cell (heterokaryon). If the nuclei in a heterokaryon fuse, the fused nucleus is referred to as a synkaryon, and the cell is known as a hybrid (Freshney, 2005). Cell fusion occurs spontaneously at low levels in culture, and its frequency can be increased by treating cells with a fusogenic agent (Davis, 1994). There are three methods commonly used to induce cell fusion. The first cell fusion experiments used inactivated Sendai virus (Freshney, 2005). Although this method is easy and convenient, it was rapidly superseded by polyethylene glycol-mediated fusion, largely due to difficulties in obtaining Sendai virus (Davis, 1994). Polyethylene glycol (PEG) is still a common method to fuse cells, because of its ease of use and reliability. The concentration of PEG is critical, as it is toxic to cells.

Cells can also be fused by application of an electric field. Electrofusion occurs, provided that the electropermeabilised membranes of the cells are brought into close contact (Neil and Zimmermann, 1993; Teissie and Ramos, 1998). Then, selection techniques are used to identify the hybrid cells which often involve the

use of genetic markers in one or both parental cells (Davis, 1994; Sasaki *et al.*, 1996).

2.8.4 Suppression of Apoptosis

The finite life span of cells in culture is regulated by a group of dominant senescence genes, the products of which negatively regulate cell cycle progression (Sasaki *et al.*, 1996). Disrupting those genes, which are involved in limiting the proliferative potential of cells, should allow escape from the senescence pathway and push cells towards immortalisation and/or neoplastic transformation (Crane, 1999).

Many viruses possess genes encoding proteins that suppress or delay apoptosis long enough for the production of viral progeny (Teodoro and Branton, 1997). Small DNA tumour viruses are able to induce DNA synthesis in quiescent host cells, activating the synthetic machinery of the host cell DNA to replicate viral DNA. Adenovirus, SV40, polyomavirus, and human papillomaviruses (HPV) have evolved strategies to evade or delay apoptosis (Teodoro and Branton, 1997; O'Brien, 1998). Each of these viruses encodes proteins that interact with key regulators of the cell cycle to stimulate unscheduled DNA synthesis. Many of these proteins operate by inactivating a number of cell cycle regulatory genes, such as protein 53 (p53) and the retinoblastoma (Rb) gene (Sager, 1992). For example, some viruses have developed specific strategies to block apoptosis induced by p53, a transcription factor encoded by the TP53 gene. p53 has been described as “the guardian of the genome,” referring to its role in conserving stability by preventing gene mutation (Strachan and Read, 1999). p53 acts at the first gap phase (G_1) checkpoint, ensuring the integrity of the genome prior to cells entering the synthesis (S) phase. p53 is usually inactive in somatic cells, which proceed around the normal cell cycle (Figure 2.4). In response to DNA damage, such as from UV radiation or oncogenes, p53 is activated by phosphorylation and induces activity of genes whose products result in either growth arrest at G_1/S interface or apoptosis (Sager, 1992; Levine, 1997). Thus, long-term inactivation of p53 seems to be a key to suppression of apoptosis, providing the potential for cell transformation. For example, the adenovirus, E1B 55k, induces cell

transformation by binding to and inhibiting the transcriptional activation and growth-suppression functions of p53 (Martin and Berk, 1998), causing infected cells to continually divide.

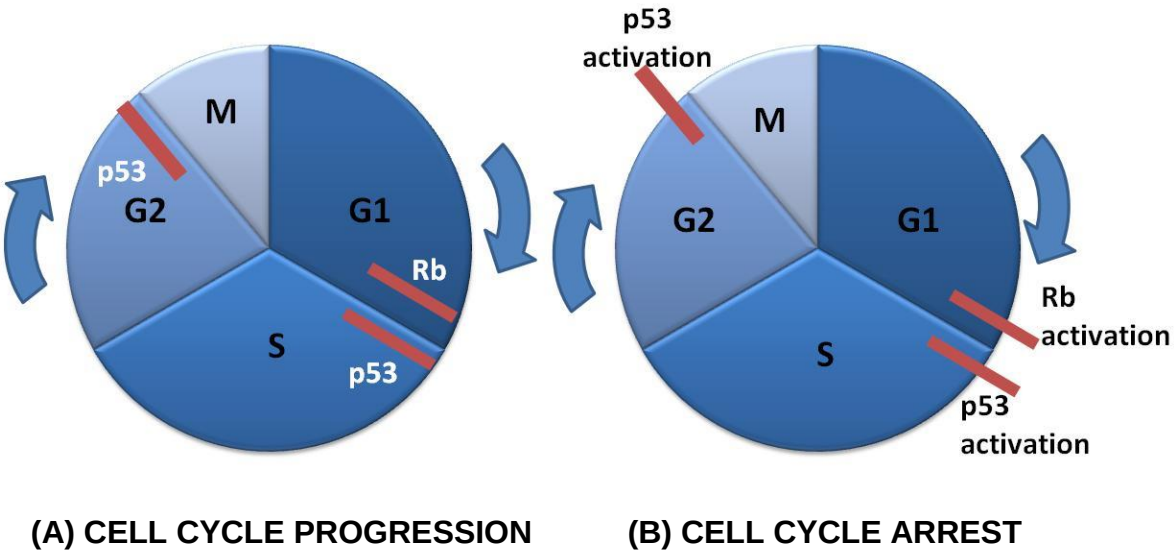


Figure 2.4. Cell cycle progression (A) and inhibition (B). The cell cycle is arrested at checkpoints by the action of inhibitors, such as p53 and retinoblastoma (Rb) gene. If p53 is activated, cells do not proceed into DNA synthesis, and mitosis does not occur (B). For continual cell cycle progression, p53 and Rb must remain inactivated. Source: Freshney, 2005.

The role of p53 *in vitro* continues to be studied extensively in mammalian cultures, because cancer cells use this pathway to evade apoptosis by inactivation. The inactivation of p53 is the most prevalent alteration found in human and animal tumours (Ko and Prives, 1996; Levine, 1997). However, its effect and activation in invertebrates, other than insects, is relatively unknown. It would be of interest to discover whether p53 is activated in crustacean cell cultures, thereby disabling the ability of cells to continue DNA synthesis.

Baculovirus genes have also been implicated in the inhibition of apoptosis: two viral genes are known to possess the ability to suppress cell suicide. Baculovirus p35 is of particular interest, since it not only blocks virus-induced apoptosis, but also functions as a general and effective suppressor of programmed cell death when expressed ectopically (Fisher *et al.*, 1999). Its broad effectiveness is related to its potent inhibition of group I, II, and III

caspases (Fisher *et al.*, 1999). The second class of baculovirus genes, inhibitors of apoptosis (IAP), also prevent apoptosis. The mechanism by which IAP proteins inhibit apoptosis is not yet understood, but they contain a conserved CH_3C_4 (RING) zinc finger at the carboxy terminus, implying a role in transcriptional regulation (Teodoro and Branton, 1997).

Another reason cells stop dividing is telomere depletion. Telomeres are protective DNA fragments situated at the ends of linear chromosomes (Colgin and Reddel, 1999). They contain repetitive DNA sequences, which become shorter with each cell division until they no longer protect the chromosome, causing the cell to die. In many cell lines, telomere maintenance is provided by the action of the ribonucleoprotein enzyme complex, telomerase (Colgin and Reddel, 1999). Telomerase is expressed in germ cells and has moderate activity in stem cells, but is absent from normal somatic cells. It is also found in most tumours. Deletions and/or mutations within senescence genes can allow cells to escape from the negative control of the cell cycle and re-express telomerase (Bodnar *et al.*, 1998; Colgin and Reddel, 1999). Some cell lines have low or undetectable levels of telomerase activity, and there is evidence that these cells maintain their telomeres by an alternative mechanism (Colgin and Reddel, 1999). For example, telomere maintenance in *Drosphilia melanogaster* does not depend on the action of telomerase (Siriaco *et al.*, 2002). Instead, two families of non-long terminal repeat (LTR) retrotransposable elements maintain telomere length by transposing specifically to chromosome ends (Levis *et al.*, 1993; Mason and Biessmann, 1993). This mechanism may also occur in crustacean cells; however, telomere extending mechanisms have not been fully investigated in crustacean cells.

In addition to telomerase, telomere-binding proteins may regulate telomere length. There is an increasing list of proteins reported to bind to the telomere and/or affect telomere length, such as TRF1, TRF2 and UP1 (Colgin and Reddel, 1999). Telomere-binding proteins may affect telomere maintenance by modulating the activity of telomerase, or by protecting chromosome ends against loss of telomere repeats.

2.8.5 Cloning

A clone is a population of cells descended from a single parental cell (Davis, 1994). Cloning is achieved by isolating a single cell and allowing it to multiply to produce a large number of cells, giving rise to a pure population. Cloning minimises the degree of genetic and phenotypic variation within a cell population. Cloning may be particularly useful in primary cell cultures, where isolating specific cell types from a mixed population can stop unwanted cell types, such as fibroblasts, from overgrowing the culture. A variety of cloning techniques have been developed, most of which are simple and effective. Serial dilution is one of the more common and less technically demanding techniques. This cloning method was used to establish a (non-verified) permanent cell line of the crayfish, *Orconectes limosus* (Neumann *et al.*, 2000). Other techniques include colony formation in semi-solid media (agar) and separation of cells by fluorescence-activated cell sorting (Freshney, 2005).

Cloning of cells can be used to isolate cells with properties closely resembling those of the original population, or to isolate cell variants. For example, cells transfected with DNA do not always have homogeneous genetic constitutions, and cloning of individual cells allows selection of cells with desirable characteristics. Therefore, cloning is useful in combination with transformation.

2.9 CRITICAL ANALYSIS OF CRUSTACEAN CELL LINE DEVELOPMENT

Researchers have faced many obstacles in pursuit of the development of a crustacean cell line. Invertebrate cells pose different challenges than their vertebrate counterparts. Whilst many invertebrate species have been used in the establishment of permanent cell lines, no verified aquatic invertebrate cell line exists, despite numerous attempts. Whether the difficulties of establishing a crustacean cell line are due to cellular aetiology or a culture environment deficit is under debate. This section discusses the factors that have plagued the development of crustacean cell lines and ways to potentially combat them.

2.9.1 Cell Physiology and Culture Media

Little is known about the *in vitro* physiology of aquatic invertebrate cells. Consequently, few appropriate culture media and culture conditions have been developed. This lack of information may explain the low frequency of mitosis observed in crustacean cell culture. Most studies reported that crustacean cells survived in the culture medium, but few reported replication of cells *in vitro* (Mothersill and Austin, 2000). This suggests that some element of the culture medium is lacking, or that a required culture condition is not being met, causing the cells to enter a stationary growth phase. In studies where mitosis was observed, mitotic division often occurred in the first week of culture and declined over the culture period (Luedeman and Lightner, 1992; Neumann *et al.*, 2000; Mulford *et al.*, 2001; Fan and Wang, 2002; Lang *et al.*, 2002a; Lang *et al.*, 2002b; Maeda *et al.*, 2003). Therefore, media supplements should be investigated to identify specific growth requirements of crustacean cells *in vitro*.

2.9.2 Vertebrate vs. invertebrate cell lines

The lack of significant progress in marine invertebrate cell culture may be related to the inappropriate application of culture conditions used for vertebrate cell lines to invertebrate cell culture (Frank *et al.*, 1994; Bayne, 1998). Many scientists initially believed that cells of different taxa within the kingdom Animalia, have similar nutrient requirements, are controlled by the same developmental and biochemical pathways, and are under the expression of the same genes (Rinkevich, 1999). However, many studies have shown that this is not the case. For example, invertebrate cell surface specificities, such as lipidic and extracellular matrices, differ significantly from those of the vertebrates (Goodwin, 1991), which affects their ability to grow and attach *in vitro*. Aquatic invertebrate cell cultures appear to require approaches and involve the need to overcome obstacles not encountered in development of vertebrate cell cultures (Rinkevich, 1999). In comparison to mammalian techniques, culture methods for invertebrates are underdeveloped. Aquatic invertebrates include an enormous range of phyla, species, and cell types, and detailed knowledge of their nutritional requirements is fragmentary (Mothersill and Austin, 2000). Ultimately, the approach necessary to develop a crustacean cell line may be completely different from methodologies used for vertebrate culture.

There has been a lack of synthesis of the knowledge regarding crustacean cell culture. Many studies have selected different species or tissues to culture *in vitro*, and/or used different culture media or conditions, making comparison among studies difficult. This lack of synthesis has been a major limitation to the progression of crustacean cell culture. Crustacea includes more than 30,000 species (Mothersill and Austin, 2000). Even within species, variations at the cellular level are known to exist due to differing diets and reproductive cycles (Najafabadi *et al.*, 1992). Comparison of cultured crustacean cells using different species, tissues, and conditions, has led to conflicting information, rather than resolutions.

2.9.3 Cell Sensitivity

Whilst many researchers have successfully produced primary cell cultures, few have been able to sub-culture cells. The ability to detach and reattach cells at passage appears to be a major problem in crustacean cell culture (Fraser and Hall, 1999). Mechanical lifting of cells has been used successfully, but is an extremely delicate task (Owens and Smith, 1999; Toullec, 1999). Once cells become detached by mechanical means, there is a significant drop in re-attachment, and non-attached cells die within 1-2 days (Owens and Smith, 1999). Mulford *et al.,* (2001) attempted to enhance cell attachment with several additives, including collagen, poly-L-lysine, gelatin, concanavalin A, and fibronectin, but no enhancement of cell replication was observed. Trypsin, which is commonly used to detach mammalian cells in culture, seems to be too potent for crustacean tissues (Owens and Smith, 1999; Toullec, 1999). Newly developed enzymatic cell detachment solutions, such as accutase, or non-enzymatic formulations, such as Cellstripper ®, may be less destructive, but have not been tested with crustacean cells.

2.9.4 Cellular Senescence and Apoptosis

The cell cycle is made up of two major phases: interphase and mitosis (Figure 2.5). Growth cycle duration varies with species and cell type. Vertebrate somatic cells have a finite life span of approximate 20-100 generations (Freshney, 2005). Progression of the cell cycle can be regulated by signals from the environment. For example, low cell density leaves cells with free edges and renders them capable of spreading (Freshney, 2005), which permits their entry into the proliferation cycle if conditions are optimal (presence of growth factors, hormones, etc).

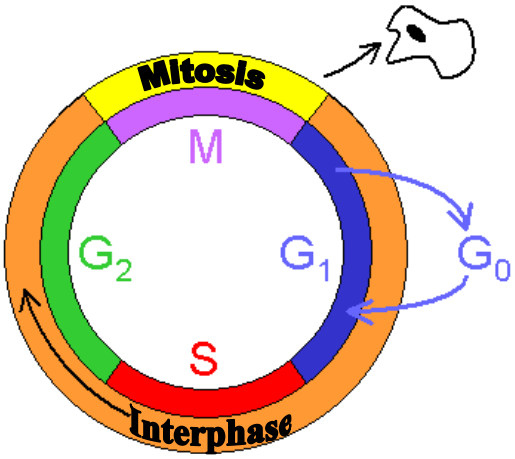


Figure 2.5. Schematic representation of the cell cycle. The cell cycle consists of two main phases. During interphase, the cell grows and replicates its genetic material. Interphase is separated into three stages: Gap 1 (G1), the gap between mitosis and synthesis; DNA synthesis (S); and Gap 2 (G2), the gap between synthesis and mitosis. Mitosis (M) is where cell division occurs. A cell may pause during G_1, prior to DNA synthesis, and enter a state of dormancy (G_0), which is sometimes irreversible.

The growth pattern of cultured cells typically consists of an initial lag phase, a period of logarithmic growth, and a stationary phase (Davis, 1994). This pattern is repeated every time the cells are sub-cultured, until somatic cells reach a

stage of senescence in which they have limited ability to divide (Figure 2.6). In senescence, the ageing cells become enlarged and flattened, and cell division is arrested at the G1/S boundary of the cell cycle (G_0). This stage appears to be the common endpoint for crustacean primary cell cultures.

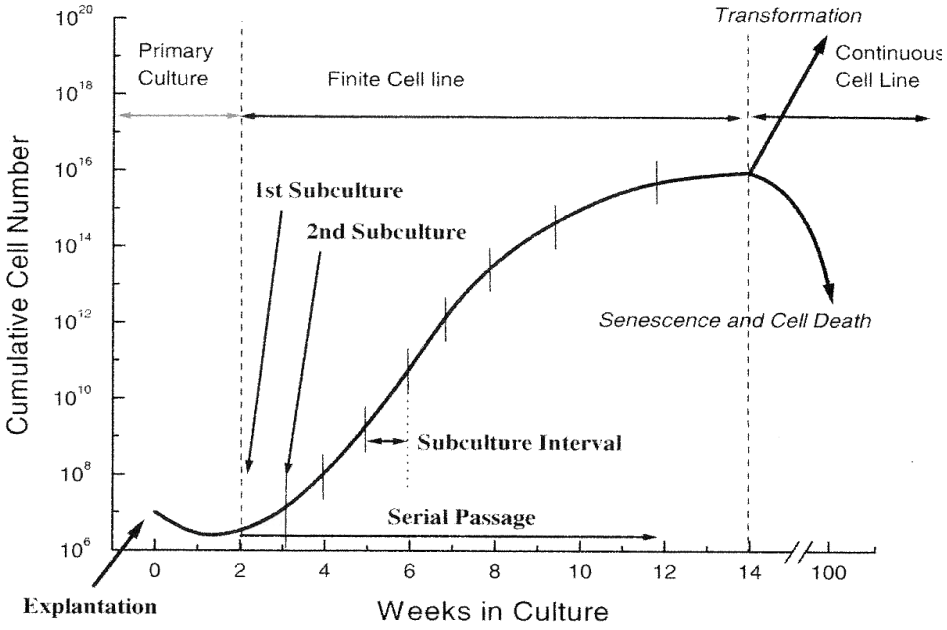


Figure 2.6. Progression of a typical cell culture. Cell number increases over time, until senescence is reached. Senescence can occur at any time, depending on the cells and culture conditions. Spontaneous transformation may occur and cause the cell culture to become immortal (Source: Freshney, 2005).

At senescence, the lag phase becomes longer with each sub-culture, and cell division slows and ultimately stops. The typical steps of senescence, as described by Crane (1999) include

1) initial phase of rapid proliferation,
2) decline in growth rate,
3) decline in saturation density,
4) complete loss of division potential,
5) enlarged and flattened cellular morphology, and
6) cell cycle arrest at G_1 (G_0).

Replicative senescence could result from inactivation of proliferation-promoting genes and/or activation of anti-proliferation genes, a decrease in the level of DNA methylation, telomere shortening, and/or activity of DNA damage and repair mechanisms (Crane, 1999). The G_1 phase of the cell cycle appears to be most susceptible to growth arrest and most important with respect to the cell's decision to divide or not. In immortal cell lines, susceptibility to arrest at the G_1 phase of the cell cycle is severely decreased (Crane, 1999). However, once DNA synthesis has been initiated past the G_1 arrest point, the cell completes the cycle.

Cells that do not exhibit senescence develop the potential to be sub-cultured indefinitely and are considered immortal (Crane, 1999). As mentioned previously, this can occur spontaneously in cell cultures or can be caused by a transforming agent. However, cultured cells can only be transformed if they have progressed beyond G_1 stage and are undergoing synthesis (S phase). There have been limited observations of continuous mitosis in crustacean cell cultures to date. This is a major obstacle in the establishment of a cell line, as cells need to synthesise DNA for transformation to occur. Determination of optimal culture conditions is necessary to enable cells to enter the DNA synthesis stage, and then potential transforming agents can be tested for their ability to delay or suppress cellular senescence and apoptosis.

2.10 CONCLUSION

Whilst many researchers have attempted to establish crustacean cell lines, many attempts have not utilised novel technologies that have been successfully applied to other invertebrate and vertebrate cell cultures. Extending the longevity of primary cell cultures is probably the most exciting and useful progress from crustacean cell culture research to date, and has come about through experimentation with cell culture additives and supplements. More work is needed in this area to identify the physiological and biochemical needs of crustacean cells, and to identify any specific growth factors required by crustacean cells. Further optimisation of culture media should enable more cell division. Meanwhile, viable short-term cultures are increasing in popularity, and

standard methodologies are beginning to appear for many penaeid species and cell types. These cultures are suitable for trialling new transfection methods that may successfully transform primary cultures into immortal cell lines.

Permanent *in vitro* models for crustacean species will allow pathogenic identification and observation of pathogen-host interactions, knowledge that will enhance health protection and disease management in crustacean aquaculture. The increasing economic importance of crustacean aquaculture encourages the development of *in vitro* methods, not only for disease management, but also for use in biotechnology, toxicology, endocrinology, and physiological studies. Through improved understanding of crustacean pathogens and mechanisms to control them, crustacean aquaculture will continue to expand, lessening the impact on wild crustacean populations that are overfished.

CHAPTER 3
GENERAL METHODS AND MATERIALS

The general methods and materials mentioned in this chapter involve techiques that have been used in more than one thesis chapter. Methods and materials used only once are contained in the appropriate chapter.

3.1 DNA Extraction

DNA was extracted from cells used in various experiments with a High Pure PCR Template Preparation Kit (Roche Sciences, Brisbane, Australia), according to the manufacturer's instructions. Briefly, the sample was lysed by incubation with the manufacturer's lysis buffer and proteinase K, to break open cell membranes and expose DNA and RNA. Binding buffer was added to inactivate nucleases, and the solution transferred to glass fibre filtered spin tubes and briefly centrifuged. After three washes with different buffers, the purified nucleic acid bound to the glass fibre filter was eluted into a sterile 1.5 ml microcentrifuge tube. Eluted DNA was stored at -20°C.

3.2 Determination of DNA Concentration

The concentration of DNA was determined by spectrophotometry. A 50 µl aliquot of each sample was dispensed into a sterile Eppendorf cuvette. Absorbance readings were recorded at wavelengths of 260 nm (A_{260}) and 280 nm (A_{280}), using an Eppendorf biophotometer (Eppendorf, Sydney, Australia) with a 1cm path length. The concentration of nucleic acid in the sample was calculated from A_{260}, based on the assumption that 1 absorbance unit is equivalent to 50 µg/ml dsDNA. The A_{260}:A_{280} ratio provided an estimate of the purity of the nuclei acid, with values of 1.8-2.0 representing pure preparations (Sambrook *et al.*, 1989).

3.3 PCR Product Analysis and Gel Purification

To analyse PCR products, 10 µl of PCR reaction mixture was used for electrophoresis on 1.5% agarose gels containing ethidium bromide at a concentration of 1mg/ml. The gels were visualised under ultraviolet transillumination.

Products selected for DNA sequencing were run on a secondary agarose gel (1-2%), using 20 µl of each sample. The gel was visualised under UV light, and DNA fragment bands were excised using a clean scalpel blade and transferred to microcentrifuge tubes. DNA was purified from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, New Jersey, USA), according to the manufacturer's instructions. Purified DNA was quantified by spectrophotometry.

3.4 DNA Amplification and Sequencing

PCR products were ligated using T&A Cloning Vector Kit (RBC Taiwan), and then cloned into HIT^{TM} competent cells (RBC, Taiwan). This method allowed blue/white screening of bacterial colonies for identification of plasmids with DNA inserts (white colonies), which were selected and further amplified using *E. coli* in broth culture. After 16 h of incubation, target DNA was purified according to the protocol of Wizard plus SV minipreps (Promega, Madison, USA).

For DNA sequencing, a 10 µl aliquot for each forward and reverse sequence was sent to Macrogen Corporation (Seoul, South Korea). Sequencing results were downloaded from the Macrogen corporate website, and analysed using Sequencher software (Genecodes Corporation, Ann Arbor, USA). Analysed sequences were compared to available databases, using the Basic Local Alignment Search Tool (BLAST) to determine approximate phylogenetic affiliations.

3.5 Primary Cell Culture Procedure and Media

To establish cell cultures, ten juvenile *Cherax quadricarinatus* (5–8 cm long) were anesthetized by immersion in ice water for 5 min, then transferred to 2.5% formaldehyde for 10 min to rid them of external contamination. Animals were further surface-sterilized with 70% ethanol, washed briefly in sterile deionised water before the hematopoietic tissue was extracted, and dissected into small pieces (~1 mm^3) using crossed scalpel blades in a laminar flow hood. Tissue fragments were then homogenized in 1.5 ml tubes, and enzymatically dissociated for 60 min in antibiotic holding medium using a magnetic stirrer and three fleas. The medium consisted of Leibovitz 15 (L-15) culture medium

(BioWhittaker, New Jersey, USA), with 4× antibiotic mix (200 IU/ml penicillin, 200 µg/ml streptomycin, 80 µg/ml kanamycin, 5 µg/ml polymyxin B, and 2.5 µg/ml fungizone®) and dispase (2.2 U/ml). Cells were then pelleted by gentle centrifugation (500×*g* for 5 min) using an Eppendorf A-4-44 rotor. The culture medium was removed, and the pelleted cells were resuspended in fresh antibiotic holding medium and re-centrifuged. This process was repeated three times. Cells were then passed through a 40 µm cell strainer (BD Falcon, North Ryde, Australia) to remove excess debris. The cells were seeded in 96-well tissue culture plates containing 100 µl of culture medium per well (Iwaki, Tokyo, Japan) or in 25 cm^2 plastic cell culture flasks (Iwaki, Tokyo, Japan) containing 10 ml/flask, at an approximate density of at ~5×10^4 cells/ml. Unless stated otherwise, the culture medium consisted of L-15 medium (BioWhittaker, New Jersey, USA) with no L-glutamine, supplemented with 2 mM GlutaMAX™ (Gibco, Brisbane, Australia), 2× antibiotic mix and 10% FBS. After 24 h, the culture medium was replaced with an equal volume of fresh medium. Medium changes were then performed on a weekly basis. Cultures were incubated at 28°C.

3.6 Observation of Cultured Cells

Cultured cells were observed daily with an Olympus IMT-2 inverted phase-contrast microscope. To allow daily and weekly cell comparisons, digital photographs of cultured cells were captured using a USB DEC-18 microscopic eye-piece camera (World Precision Instruments, Sarasota, USA).

3.7 Cell Viability Counts

To determine the amount of viable cells, equal volumes of a cell suspension and 0.2% trypan blue solution were mixed, loaded into the counting chamber of a haemocytometer, and examined using an Olympus IMT-2 inverted phase-contrast microscope. The viable cells, which were not stained blue, were counted and computed into viable cells/ml.

CHAPTER FOUR
ASSESSMENT OF HYBRID CRUSTACEAN CELL POPULATIONS

4.1 INTRODUCTION

The lack of a standardised system for the isolation, identification, and purification of crustacean viruses is a major obstacle to the expansion of the crustacean aquaculture industry. As spontaneous and induced transformation of somatic cells is an extremely rare event (Crane and Williams, 2002), creation of hybrid cells capable of supporting the growth of crustacean viruses was attempted by fusing cells from an immortal cell line with crustacean cells. This approach was used by Dr. Katrina Roper (James Cook University), who created seven lines of hybrid cells, using irreversibly blocked immortal cell lines and primary cell suspensions from *Cherax quadricarinatus* and *Penaeus monodon* in 1999. The ability to hybridise cells was first described by Kohler and Milstein (1975). Since this time, hybridised cells have been primarily used to produce monoclonal antibodies for immunological studies. In the present study, however, cell hybridisation combined genetic material to create a continuously replicating cell culture that could also support crustacean viral growth.

One of the most important aspects of fusion experiments is confirmation of the identity of the hybrid cells. There are two pathways of DNA synthesis in cells: the salvage pathway and the *de novo* pathway. The salvage pathway depends on the enzyme, hypoxanthine guanine phosphoribosyl transferase (HGPRT). The immortal cell partners used for cell fusion were modified by eliminating this enzyme and therefore these cells cannot use the salvage pathway to produce DNA. Therefore the cells can use only the *de novo* pathway to synthesise DNA and grow. The *de novo* pathway can be blocked by the addition of aminopterin, which stops DNA synthesis and proliferation of modified HGPRT cells. Cell hybrids are created by combining modified HGPRT immortal cells with other cells (e.g. crustacean cells) that are capable of synthesising DNA using both the salvage and *de novo* pathways (Figure 4.1). Aminopterin is added to block the *de novo* pathway, so DNA synthesis can only occur via the intact salvage pathway of the crustacean cell. If cell replication occurs under these conditions, a true hybrid cell must be proliferating.

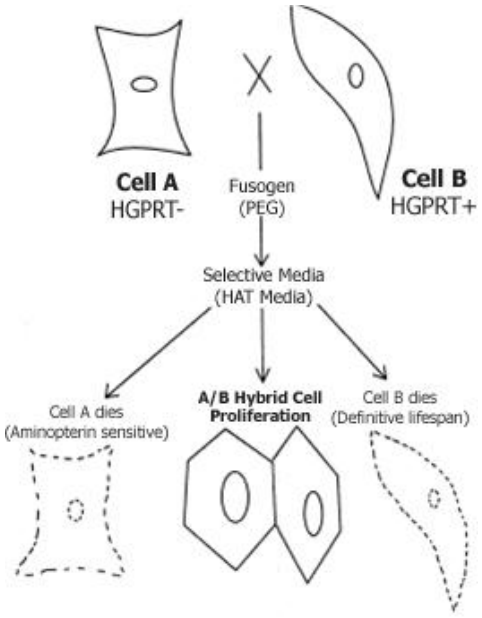


Figure 4.1. Schematic representation of cell hybridisation. Cell A is deficient in the enzyme, hypoxanthine guanine phosphorlosyl transferase (HGPRT-), and dies in medium containing hypoxanthine-aminopterin-thymidine (HAT), due to blocking of both DNA synthesis pathways. Cell B has a limited lifespan, but contains an intact salvage pathway. When cell B is fused with cell A, DNA synthesis will occur, allowing cell proliferation.

The aim of the present study was to assess the viability of, and to devise methods to confirm the presence of crustacean cellular components in the hybridised cells created by Dr. Roper. If the cells do indeed contain crustacean genes, these cells will be the most likely candidates for expression of crustacean viruses *in vitro*.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture

The hybrid cell populations from *Cherax quadricarinatus* and *Penaeus monodon* developed by Dr. Roper were frozen and stored in liquid nitrogen. For retrieval, vials of cells were taken from liquid nitrogen, quickly defrosted in a 37°C water bath, and pelleted by gentle centrifugation (500 × *g* for 7 min). The supernatant was decanted, and the pellet was resuspended in culture medium. The resuspended cells were transferred to a 25 cm^2 flask, with 10 ml of culture

medium supplemented with 10% FBS. The cells were monitored daily for growth, and once a monolayer formed, the cells were trypsinised, split, and re-seeded. Viable cells counts were conducting according to the procedure described in Section 3.7.

One clone of each of the seven hybrid cell lines (Table 4.1), and three control cell lines - fathead minnow (FHM), epithelioma papulosum cyprinid (EPC) and *Spodoptera frugiperda* pupal ovarian tissue (SF-9) - were used to investigate the presence of crustacean genes and proteins. Hybrid cell lines F12, F13, F17 and EPC cells were grown in L-15 culture medium. Fathead minnow cells and derived hybrids F14, F15 and F16 were grown in Dubecco's minimum essential medium (DMEM), and the insect SF-9 cells and hybrid F11 cells were grown in TC-100 culture medium. All cell culture media were supplemented with 10% FBS.

Table 4.1. Previously developed hybrid cell lines and media used for culture.

Sample	Cell Hybrid	Culture Medium	Number of Clones
F11	SF-9 cells (moth) × *Penaeus monodon* haemocytes	TC-100 + 10% FBS	52
F12	EPC × *Penaeus monodon* haemocytes	L-15 + 10% FBS	6
F13	EPC × *Penaeus monodon* haemocytes	L-15 + 10% FBS	20
F14	FHM × *Cherax quadricarinatus* haemocytes	DMEM + 10% FBS	6
F15	FHM × *Cherax quadricarinatus* ovaries	DMEM + 10% FBS	11
F16	FHM × *Cherax quadricarinatus* epithelial cells	DMEM + 10% FBS	5
F17	EPC × *Cherax quadricarinatus* ovarian pellet	L-15 + 10% FBS	7

4.2.2 18S rRNA PCR

To verify that crustacean DNA was incorporated into the hybrid cells, PCR was conducted using arthropod specific 18S rRNA primers 143F/143R (Lo *et al.*, 1996). Once a confluent monolayer of cells had formed, cells were trypsinised and pelleted using slow centrifugation (500 × *g* for 5 min). DNA was extracted from the cells (Section 3.1) and quantified by spectrophotometry (Section 3.2).

For PCR analysis, 1 µl of the DNA template (50-150ng) was added to 49 µl of PCR reaction mixture. PCR reaction mixture consisted of MBI fermentas PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20), 200 µM dNTPs, 1 µM of each primer, 1.5 mM $MgCl_2$, and 1U *Taq* polymerase (MBI fermentas, Hanover, USA). The arthropod primer sequences were 143F, 5' – TGC-CTT-ATC-AGC-TAC-GTT-CGA-TTG-TAG-3' and 145R, 5'-TTC-AGA-CGT-TTT-GCA-ACC-ATA-CTT-CCC-3'. Amplification was carried out in an Eppendorf Mastercycler gradient; expected amplicon size was 848 bp. The PCR profile consisted of 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, and a final 5 min extension at 72°C. A DNA extract of *Penaeus monodon* tissue was used as a arthropod-positive control, and the negative control contained no DNA template. PCR products were visualised by electrophoresis (Section 3.3). Amplicons with the expected size of 848 bp were excised from the gel, purified, and sent for DNA sequence analysis (Section 3.4).

4.2.3 Haemocyanin PCR

To confirm the presence of crustacean genetic material in the hybrid cells, identification of the gene for haemocyanin was attempted. Haemocyanin is a blue copper-containing oxygen transport molecule that is a predominant protein in crustacean haemolymph (Durstewitz and Terwilliger, 1997). The same DNA extracts used for the arthropod 18S rRNA PCR were used. To test the specificity of the primers, DNA was also extracted from the pleopods of six other crustacean species: *P. merguiensis*; *P. vannamei*, *P. stylirostris, Portunis pelagicus*; *Scylla serrata*, and *Macrobrachium rosenbergii*. DNA was extracted as previously described (Section 3.1). Primers for the haemocyanin gene were designed on the *P. monodon* haemocyanin gene sequence (AF431737)

(Terwilliger *et al*., 1999). No DNA sequence currently exists for the haemocyanin gene of *C. quadricarinatus*.

For PCR analysis, 1 µl of the DNA template (50-150 ng DNA) was added to 49 µl of PCR reaction mixture. PCR reaction mixture consisted of MBI fermentas PCR buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20) 200 µM dNTPs, 1 µM of each primer, 1.5 mM $MgCl_2$ and 1 U *Taq* polymerase (MBI fermentas, Hanover, USA). The haemocyanin primer sequences were H-299, 5' –TCG-ACA-GGG-CTG-GTA-ATC-ATA-3' and H-594, 5'-CCG-GCA-AAT-GTT-AGT-TCT-3'. Amplification was carried out in an Eppendorf Mastercycler; expected amplicon size was 295 bp. The PCR profile consisted of 3 min denaturing at 95°C, followed by 40 cycles of 95°C for 1 min, 59°C for 1 min, and 72°C for 1 min, with a final 5 min extension at 72°C. DNA extracted from *P. monodon* haemocytes was used as a positive control; the negative control contained no DNA template. PCR products were visualised by electrophoresis (Section 3.3). Amplicons with the expected size of 295 bp were excised from the gel, purified, cloned, and sent for DNA sequence analysis (Section 3.4).

4.2.4 Immunohistochemistry Using Monoclonal and Polyclonal Antibodies

A monoclonal antibody (MAb) developed by Ainsworth (2000) was used to assess the presence of crustacean proteins in hybrid cell populations. After assessing contamination and responsiveness of the antibodies, antibody 1.9B2-5 was selected for immunostaining of the selected hybrid cell lines. Antibody 1.9B2-5 was categorised as a type I antibody with general haemocytic specificity, staining approximately 78% of *P. monodon* haemocytes (Ainsworth, 2000).

Polyclonal antibodies (pABs) were also used to assess crustacean proteins in the hybrid cell populations. In contrast to monoclonal antibodies, which are derived from a single cell line, a PAb contains a mixture of immunoglobulin molecules secreted against a specific antigen. The polyclonal antibodies used in the present study were initially developed in chickens against gill associated virus (GAV) of *P. monodon* (Munro and Owens, 2007). This particular PAb was

found to lack reactivity against GAV and was instead concluded to have a presumptive prawn cell origin which therefore may contain an array of non-specific proteins that react with *P. monodon* cells, making it an ideal candidate to use against the developed *P. monodon* hybrid cells.

For immunostaining of both the MAb and PAb, cells were spun onto positively charged microscope slides and fixed in acetone for 20 min. Endogenous peroxidase activity was blocked by adding 0.3% hydrogen peroxide in methanol to each slide for 30 min. After washing with PBS-T (Appendix 2), slides were incubated with either the MAb or PAb (1:100) for 1 h at 37°C in a humid enclosed chamber. Slides were subjected to three 5 min washes with PBS-T, and incubated with the secondary antibody (goat anti-mouse HRP conjugate, 1:120, Jackson Immuno Research, West Grove, USA) in a humid chamber for 1 h at room temperature. Slides were again subjected to three 5 min washes with PBS-T. Slides were then incubated with fresh diaminobenzidin substrate for 10 min, washed briefly in water, and counter-stained with haematoxylin for 2 min. Slides were again washed in water, then exposed to Scott's tap water substitute for 30 s before a final water wash. Slides were then dehydrated through grades of ethanol, cleared with xylene, and mounted. Slides were microscopically examined using an Olympus BH-2 microscope.

4.2.5 ELISA

For quantitative analysis of crustacean proteins, an indirect ELISA was developed using chicken anti-GAV polyclonal antibodies (Munro and Owens, 2007). Hybrid cells were trypsinised from 25 cm^2 flasks and centrifuged at 500 × *g* for 5 min. The cell count was determined by haemocytometer. Approximately 1×10^6 cells were lysed by adding 125 µl of CellLytic MT reagent (Sigma, Brisbane, Australia). After 15 min of incubation with lysis reagent, cells were centrifuged at 15,000 x *g* for 10 min, and the protein-containing supernatant was removed for use as an antigen in the ELISA. Cell supernatant was diluted (1:1) in carbonate coating buffer (TropBio, Townsville, Australia), and 50 µl were loaded into each well of a 96-well U-bottom plate (Sarstedt, Adelaide, Australia). The plate was incubated overnight at room temperature in a humid environment. Following coating, 150 µl of post-coating buffer (TropBio

Townsville, Australia) were added, the plate was incubated for 1 h and washed three times in wash buffer (Trop Bio, Townsville, Australia). Fifty µl of diluted (1:80) PAb were added to each well, incubated for 1 h at room temperature in a humid environment and washed three times with wash buffer. The secondary conjugated antibody was diluted (1:120), and 50 µl were added to each well. Following 1 h incubation at room temperature, the plate was washed six times with wash buffer. Finally, 100 µl of 2,2′-azino-di-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (KPL Europe, Guilford UK) was added to each well, and the plate was again incubated for 1 h. Colour formation was determined using a Labsystems Multiskan EX ELISA plate reader (Pathtech, Preston, Australia).

A secondary ELISA was run to detect the crustacean protein, tropomyocin. Two samples were tested: one sample of a *P. monodon* hybrid (F12), and one of a *C. quadricarinatus* hybrid (F16). Samples were sent to Elisa Systems in Windsor, Brisbane, for subsequent ELISA analysis.

4.3 RESULTS

4.3.1 Cell Culture

All cell lines contained viable cells upon resuscitation from liquid nitrogen. Some clones were contaminated upon seeding; these clones were discarded and a new clone from the same hybrid was used as a replacement. Growth characteristics of the cells varied among cell lines, and 3-7 d was typically required for a complete monolayer to form (Figure 4.2).

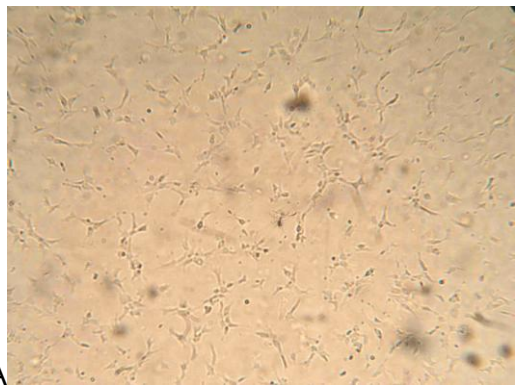
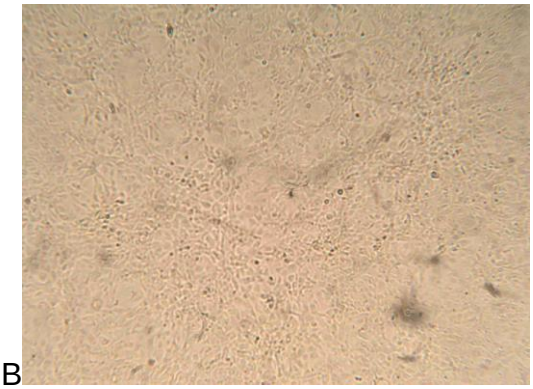
A  B 

Figure 4.2. (A) Cell growth of hybrid F12-clone C5 (F12C5) after 24 h. (B) Monolayer formed from F13-clone G10 (F13G10) after 72 h.

4.3.2 18S rRNA PCR

All hybrid cell lines produced PCR amplicons of varying intensity at the expected size of 848 bp, using primers for the arthropod 18S rRNA gene (Figure 4.3).

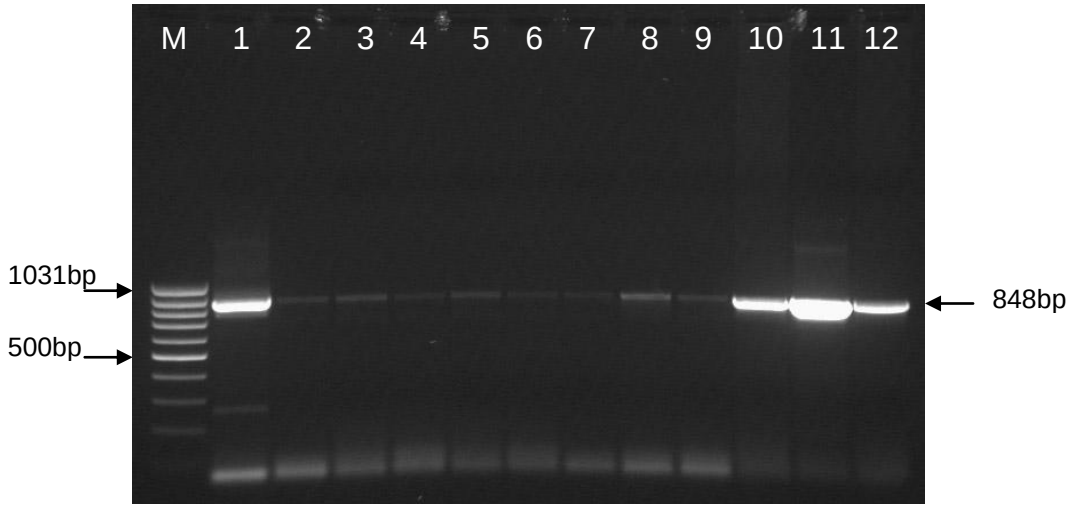


Figure 4.3. PCR for the arthropod 18S rRNA gene in cell hybrids F11-F17, control cell lines, and haemocytes from *C. quadricarinatus* and *P. monodon*. Lane M: GeneRuler; Lanes 1-7: Cell hybrids F11-F17; Lane 8: FHM cells; Lane 9: EPC cells; Lane 10: SF-9 cells; Lane 11: *C. quadricarinatus* haemocytes; Lane 12: *P. monodon* haemocytes.

Sequencing of the cell hybrids showed no evidence of crustacean 18S rDNA, but did contain 18S rDNA from the fish or insect hybrid partner. Amplicons from *P. monodon* and *C. quadricarinatus* haemocytes matched 99% and 97%, respectively, to the 18S rDNA sequences for those crustaceans listed in the BLAST database.

4.3.3 Haemocyanin PCR

PCR results showed that all hybrid cells were negative for the crustacean haemocyanin gene (Figure 4.4). A PCR-positive amplicon was obtained using DNA extracted from *P. monodon* haemocytes.

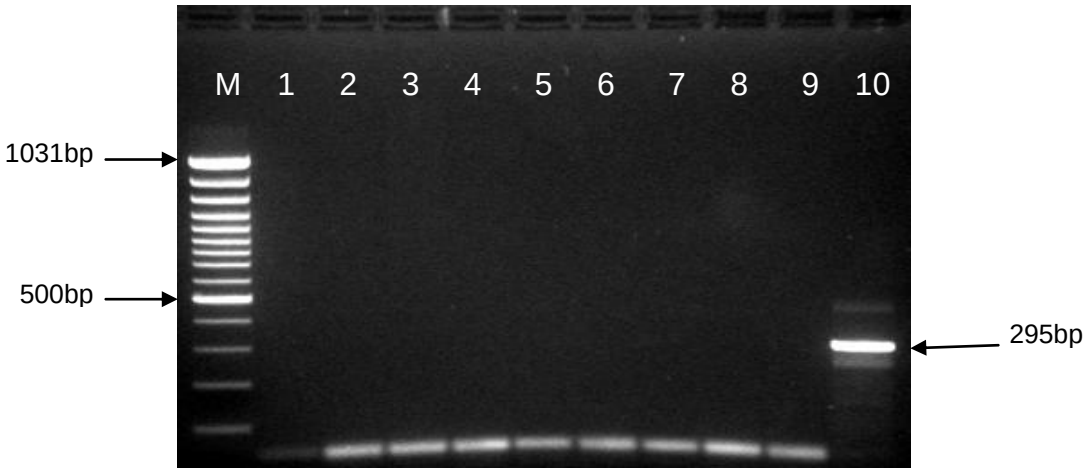


Figure 4.4. PCR for the crustacean haemocyanin gene in hybridised cells. No amplification was observed. Lane M: GeneRuler; Lanes 1-7: Cell hybrids F11-F17; Lane 8: No template control; Lane 9: *C. quadricarinatus* haemocytes; Lane 10: *P. monodon* haemocytes.

Although it was hypothesised that the haemocyanin gene would be conserved among crustacean species, the haemocyanin primers were specific for *P. monodon* DNA only (Figure 4.5). DNA sequencing of the PCR-positive amplicon matched 100% to the *P. monodon* haemocyanin gene (GenBank accession no. AF431737).

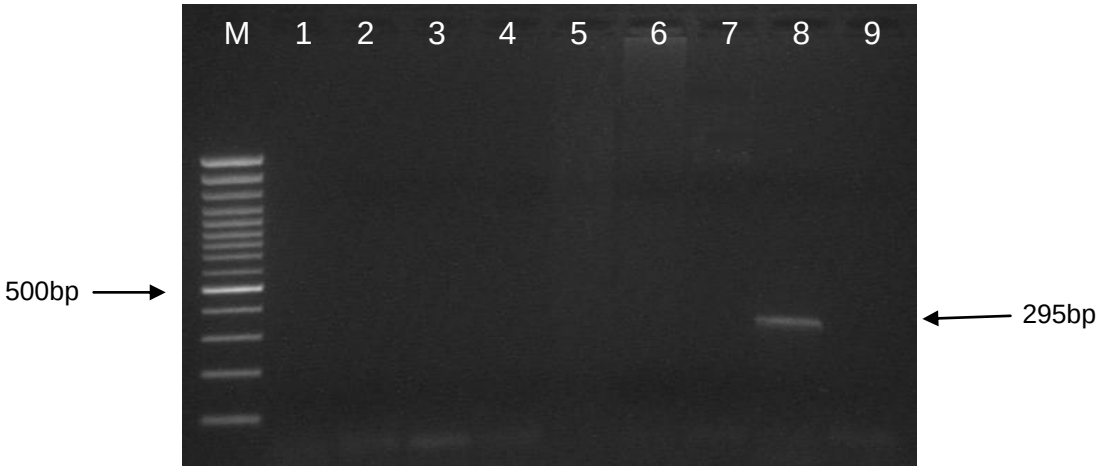


Figure 4.5. PCR for the presence of the crustacean haemocyanin gene in eight crustacean species (pleopod tissue extracts). Lane M: GeneRuler; Lane 1: *C. quadricarinatus*; Lane 2: *P. merguiensis*; Lane 3: *Portunis pelagicus*; Lane 4: *Scylla serrata*; Lane 5*: P. vannamei*; Lane 6: *Macrobrachium rosenbergii*; Lane 7: *P. stylirostris*; Lane 8: *P. monodon*; Lane 9: No template control.

4.3.4 Immunohistochemistry Using Monoclonal and Polyclonal Antibodies

No staining was present when *P. monodon* haemocytes were exposed to the monoclonal antibody 1.9B2-5, raised against *P. monodon* haemocytes (Figure 4.6A). Therefore, it was not surprising that cell populations fused with *P. monodon* haemocytes did not produce positive staining when exposed to the same MAb (Figure 4.6B).

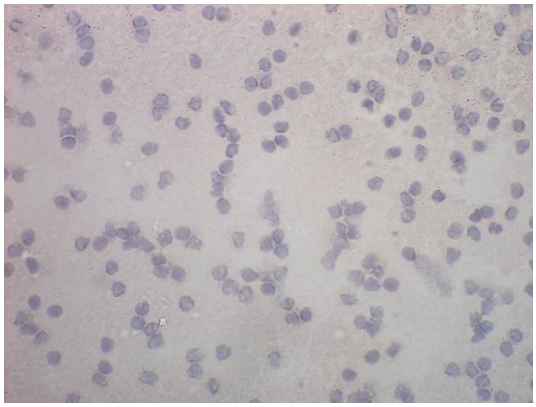
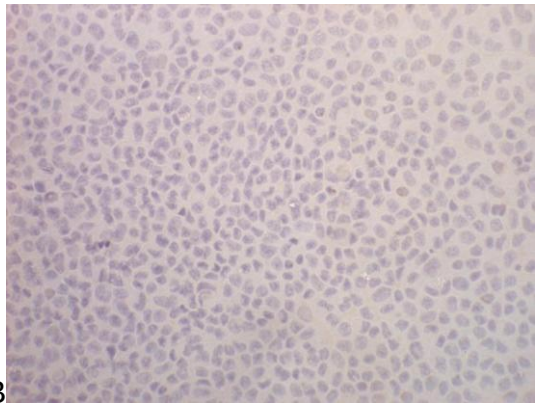
A  B 

Figure 4.6. Immunostaining with the previously developed monoclonal antibody 1.9B2-5, raised against *P. monodon* haemocytes. No staining was present with (A) *P. mono*don haemocytes or (B) cell hybrid F13G10.

Positive staining was present when *P. monodon* haemocytes were exposed to the polyclonal antibody that was initially raised against GAV of *P. monodon* (Figure 4.7A). This enabled the use of *P. monodon* haemocytes as a positive control for immunostaining with this PAb. Cell hybrids (F12 and F13) partnered with *P. monodon* haemocytes were also positive when exposed to this PAb (Figure 4.7B). However, EPC cells, which were initially included in this study as a potential negative control, also produced positive staining (Figure 4.7C), suggesting this antibody was not specific enough for detection of crustacean proteins.

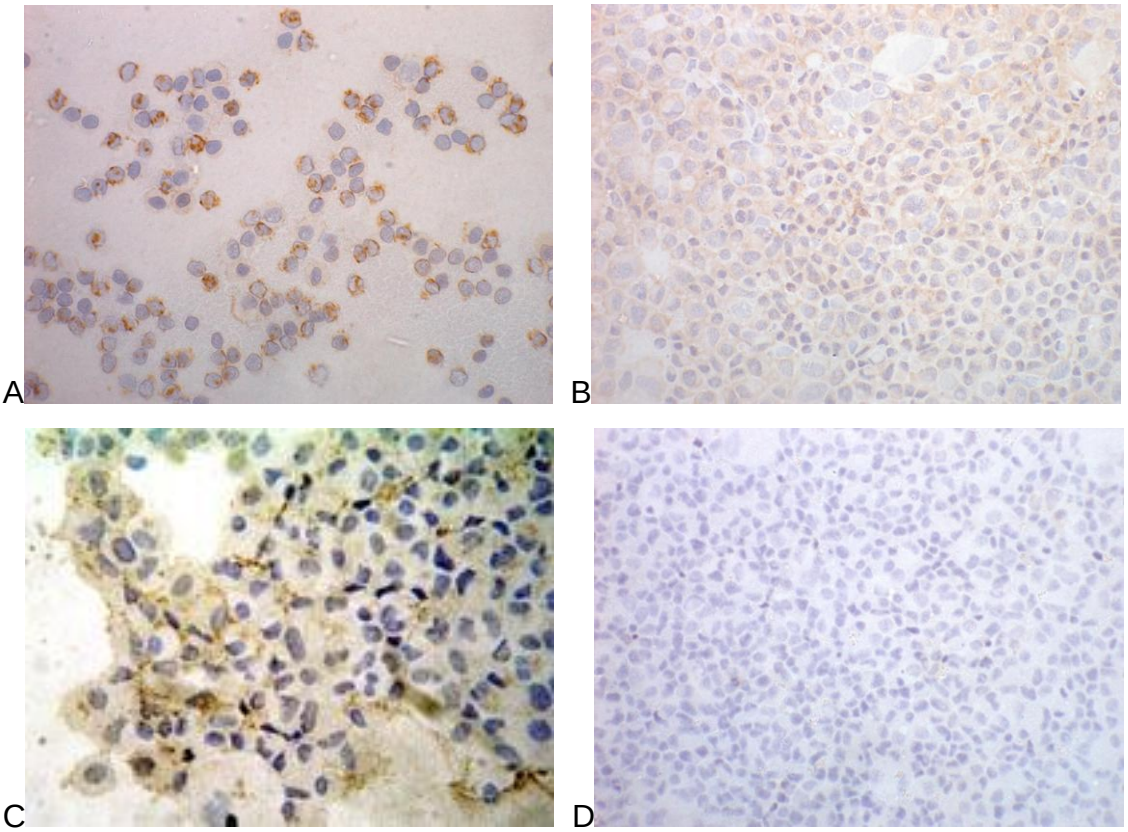


Figure 4.7. Immunostaining with a polyclonal antibody from *P. monodon* (positive control). (A) *P. monodon* haemocytes, (B) F13G10 (EPC and *P. monodon*) hybrid cells, (C) EPC cells, and (D) EPC cells not exposed to PAb (negative control).

4.3.5 ELISA

The more sensitive antibody detection method is through the use of a capture ELISA. The control EPC cells produced a higher absorbance reading than any of the hybrid cell populations (Figure 4.8). This further supports the notion of non-specificity of the PAb.

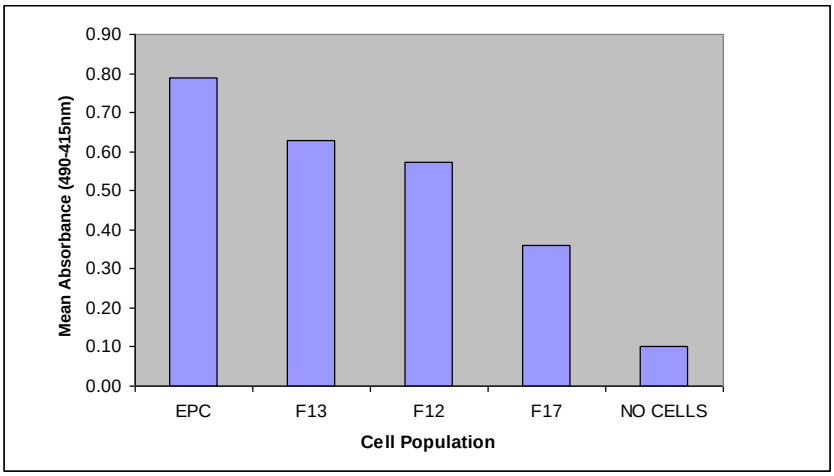


Figure 4.8. ELISA mean absorbance readings for EPC cells and EPC hybrid cell lines.

Results of the tropomyocin ELISA showed that cell hybrids F12C5 (*P. monodon* hybrid) and F16F6 (*C. quadricarinatus* hybrid) were negative for the crustacean protein, suggesting a lack of tropomyocin expression the hybrid cells.

4.4 DISCUSSION

4.4.1 Genomic Approach

Investigation into the expression of the arthropod 18S rDNA gene showed that all cell hybrids, along with parental immortal cells, were PCR-positive. However, DNA sequencing indicated that the amplicons were related to the parental fish 18S rRNA gene. Although the primers were developed for specific detection of arthropod 18S rRNA gene, slight mismatches in primer regions allowed successful amplification of fish 18S rRNA gene. This prevented the use of this gene and PCR system as a detection method for crustacean cellular components in hybrid cell populations. Therefore, a more specific gene was needed for identification of crustacean material. Haemocyanin is the primary transporter of oxygen in crustacean and mollusc species. Primers for PCR detection were developed based on the *P. monodon* haemocyanin DNA sequence. Therefore, subsequent PCR was only able to detect the presence of this gene in *P. monodon* cells. Hybrid cells that were fused with *P. monodon* haemocytes (F11, F12, F13) were the only samples with potential for a positive

PCR result. However, none of these hybrids showed PCR amplification for this gene, indicating the *P. monodon* haemocyanin gene is not present.

4.4.2 Protein Approach

Numerous immunohistochemisty trials using different concentrations of primary and secondary antibody, 1.9B2-5, resulted in no positive staining of *P. monodon* haemocytes or any of the hybrid cell populations. This antibody may have lost its ability to react; as incorrect storage of antibodies can lead to their degradation and change their affinity parameters (Dmitriev *et al.*, 2001).

Although the PAb used in the present study was initially raised against GAV of *P. monodon*, cross-reactivity of other antibodies were demonstrated with *P. monodon* haemocytes. However, this reactivity also occurred in non-crustacean EPC cells, suggesting the antibodies were reacting to a cellular membrane protein, rather than to a specific crustacean protein. During the original purification of the GAV, sucrose-purified bands may have contained cellular membrane proteins (Munro and Owens, 2007), which were subsequently injected into the chickens used for antibody development. The lack of specificity of some of these antibodies was confirmed by ELISA, in which EPC cells and all EPC hybrid cells reacted.

The tropomyocin detection ELISA is routinely performed to detect this crustacean protein in food products, as its presence can cause severe allergic reactions in people who are genetically predisposed (Li *et al.*, 2006). The negative results for tropomyocin suggested that this protein was not expressed in the cell hybrids, and that the gene encoding tropomyocin was either not passed on to hybrid cells or was switched off.

In summary, the make-up of the chromosomes, genes, and proteins of the hybridised cells developed by Dr. Roper remains unknown. Although cell-to-cell fusion is universally involved in processes such as fertilisation and other biological events, the mechanisms involved in fusion vary among cell types and are not fully understood (Podbilewicz and White, 1994). The deficiency of crustacean genes and proteins observed in the hybrid cells may be due to the

absence of crustacean chromosomes. Many studies of cross-species cell hybrids have found that the chromosomes of one species are preferentially lost (Lucas and Terada, 2003) or gradually lost over time (Weiss and Green, 1967). Cell membranes can blend or merge without fusion of the cellular contents (Podbilewicz *et al.*, 2006). It is possible that the only component of the crustacean cells passed on to the immortal cell partners was the intact HGPRT pathway. Further research is required to confirm the identity of Dr Roper's hybrid cells. The first step would be single cell cloning of hybrid cells, in an effort to minimise heterogeneity. Following cloning, karyotypic analysis of the parental and hybrid cells could be compared to confirm the makeup of the hybrid cells. If future hybrids were created, the inclusion of an antibiotic-resistant selection marker (e.g. neomyocin) would be a useful tool for selecting hybridised cells.

CHAPTER FIVE
VIRAL REACTIVITY IN HYBRID CRUSTACEAN CELL POPULATIONS

5.1 INTRODUCTION

Viable cell cultures derived from aquatic crustaceans would be useful for a variety of applications, including pathogen identification and potential development of disease treatments. The ability to grow crustacean viruses in culture would also provide a potentially infinite source for all types of viral research. Current investigations of crustacean viruses rely primarily on bioassays, which involve the injection of live virus into specific pathogen-free (SPF) animals, with the hope that the virus will replicate enough to permit further investigation. While this method has proven useful in a number of studies on prawn viruses (Overstreet *et al.*, 1997; Kiran *et al.*, 2002; Nunan *et al.*, 2003; Poulos *et al.*, 2006), this system of viral amplification is inconsistent and does not allow the disease process to be studied at the cellular level (e.g. viral entry and replication strategies).

To date, several investigators have demonstrated that primary cultures of prawn cells can support *in vitro* growth of certain prawn viruses: monodon baculovirus (Chen and Kou, 1989), yellowhead virus (YHV, Lu *et al.*, 1995a; Lu *et al.*, 1995b; Chen and Wang, 1999; Assavalapsakul *et al.*, 2003), whitespot syndrome virus (WSSV, Chen and Wang, 1999; Uma *et al.*, 2002; Maeda *et al.*, 2004; Jiravanichpaisal *et al*., 2006), penaeid rod-shaped baculovirus (PRBV, (synonym of WSSV, Itami *et al.*, 1999), and systemic ectodermal and mesodermal baculovirus (synonym of WSSV, Kasornchandra *et al.*, 1999). However, primary cell cultures are tedious to develop, inconsistent, and time consuming to establish and maintain. Continuous cell lines have several advantages over primary cell cultures as a research and diagnostic tool. First, immortal cell lines can be continuously passaged *in vitro*, eliminating the need to return to live animals as a source of cells. Second, cell lines are often homogeneous with respect to genotype and phenotype, exhibiting stable properties, such as virus susceptibility. Finally, cell lines can be mass cultured within a short period, which may be essential in the face of a serious and extensive disease outbreak (Crane, 1999).

Viral detection in cell culture is usually determined by the presence of cytopathic effects (CPE). However, CPE is not always an accurate method for determining the presence or quantity of virus in cell cultures, as cytotoxic effects may produce similar symptoms to CPE (Audelo-del-Valle *et al.*, 2003; Pantoja *et al.*, 2004). Furthermore, viruses can propagate *in vitro* without any observable CPE (Donofrio *et al.*, 2000).

Technology using real-time PCR allows accurate quantification of viral loading. Real-time PCR is the most sensitive and accurate quantification method currently available, utilising Taqman® probe chemistry to directly determine the number of viral copies in a sample, and eliminates the need for CPE determination. The present study utilised real-time PCR technology to determine if existing hybrid cell populations developed from crustaceans (Section 4.2.1) could support replication of penaeid viruses.

5.2 MATERIALS AND METHODS

5.2.1 Cell Culture

One clone from each of the seven hybrid cell lines (Table 4.1), plus three control cell lines of FHM, EPC, and SF-9, were used to investigate viral susceptibility. Hybrid cell lines F12, F13, F17 and EPC cells were grown in L-15 culture medium. Fathead minnow cells and their derived hybrids F14, F15 and F16 were grown in DMEM, and the SF-9 insect cells and F11 hybrid cells were grown in TC-100 culture medium. All cell culture media were supplemented with 10% FBS.

5.2.2 Crustacean Viruses

Two viruses known to infect *Penaeus* spp. were selected for inoculation of cell cultures, largely due to their prevalence and availability in Australia: hepatopancreatic parvo-like virus (HPLV, as named by the International Committee on Taxonomy of Viruses) and infectious hypodermal and haematopoietic necrosis virus (IHHNV). A more lethal virus, such as WSSV, might have been more suitable for viral inoculation of cell cultures, but this virus is currently exotic to Australia.

5.2.3 Viral Purification

Similar methods were used to isolate and purify HPLV and IHHNV from prawn tissue. For HPLV, 25 hepatopancrei were removed from HPLV PCR-positive *Penaeus merguiensis* (Elliman pers. comm.). For IHHNV, 100 pleopods were removed from 10 *P. monodon*. Hepatopancrei or pleopods were homogenised in 250 ml of TN buffer (Appendix 2) and centrifuged at 4,500 × *g* for 15 min to pellet tissue debris. The supernatant was re-centrifuged at 13,000 × *g* for 15 min. The liquid supernatant was collected and vacuum filtered through 0.45 µm filter membrane. This suspension was ultracentrifuged at 200,000 × *g* for 1 h at 4°C in a Beckman-Coulter Optima L-90K centrifuge with a 70Ti rotor. The pellet was resuspended in 3 ml TN buffer, layered onto a 20-40% discontinuous sucrose gradient, and ultracentrifuged (160,000 × *g* for 3 h) at 4°C, using a SW40Ti swing rotor. Following centrifugation, the sucrose layers were discarded, and the viral pellet was covered with 3 ml of TN buffer, covered with parafilm, and stored at 4°C overnight to allow the pellet to loosen. The following day, the viral pellet was fully resuspended, transferred to a sterile 1.5 ml Eppendorf tube, and stored at -20°C.

5.2.4 Verification of Virus

To verify the presence of viral DNA, a DNA extraction was performed on 200 µl of purified virus. For detection of HPLV DNA, one µl of DNA template was added to 49 µl of PCR master mix, which consisted of MBI fermentas PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 200 µM dNTPs, 0.5 µM of each primer, 3.0 mM $MgCl_2$, and 1U *Taq* polymerase (MBI fermentas). HPLV primer sequences were 140F 5'-CTACTCCAATGGAAACTTCTGA-3' and 140R 5'-GTGGCGTTGGAAGGCACTTC-3' (LaFauce *et al.*, 2007). Amplification was carried out in an Eppendorf mastercycler gradient, with an expected amplicon size of 140 bp. The PCR profile consisted of 94°C for 7 min, followed by 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min, with a final 5 min extension at 72°C.

For detection of IHHNV DNA, one µl of DNA template was added to 49 µl of PCR master mix, consisting of MBI fermentas PCR buffer, 200 µM dNTPs, 0.3

µM of each primer, 2.0 mM $MgCl_2$, and 1U *Taq* polymerase (MBI fermentas). IHHNV primer sequences were 392F 5'-GGGCGAACCAGAATCACTTA-3' and 392R 5'-ATCCGGAGGAATCTGATGTG-3' (Krabsetsve *et al.*, 2004). Amplification was carried out in an Eppendorf mastercycler gradient, with an expected amplicon size of 392 bp. The PCR profile consisted of 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final 7 min extension at 72°C.

Controls for the PCR tests were *P. merguiensis* tissue extract that had previously tested PCR-positive for HPLV (HPLV positive control) or *P. monodon* tissue extract that had previously tested PCR-positive for IHHNV (IHHNV positive control), and a negative control containing no DNA template. PCR products were visualised by electrophoresis (Section 3.3).

5.2.5 Viral Inoculation

Duplicate 75 cm^2 flasks of each of the seven hybrid cells, plus control cell lines of FHM, EPC, and SF-9, were grown to 60-80% confluency. The culture medium was removed, and each flask was inoculated with either 100 µl of purified virus or 100 µl of TN buffer. The flasks were gently agitated for 20 min to promote viral absorption (McClenahan *et al.*, 2005). Then, 15 ml of fresh culture medium with 5% FBS was added, without removing the inoculum. The cells were incubated at 28°C and monitored once a day for 14 d for cytopathic effect (CPE). One ml samples of cell culture medium was collected 0, 4, 7, 10, and 14 d post-exposure and replaced with equal volume of fresh medium. Two hundred µl of these samples were used to perform a DNA extraction (Section 3.1). The concentration of extracted DNA was estimated by measuring optical density, A_{260nm}, with an Eppendorf spectrophotometer (Section 3.2).

5.2.6 Real-time PCR

Quantitative real-time PCR was performed according to the protocols developed for Australian HPLV (LaFauce *et al.*, 2007) and Australian IHHNV (Krabsetsve, 2006, 2009). Assays were run with a Corbett Research Rotor Gene RG3000, using a designed Taqman® probe protocol. The reaction mixture contained 2.5 µl of DNA, 100 µM of Taqman® probe, 1.5 mM $MgCl_2$, and 25 pmols of each

primer in a final volume of 20 µl. Samples were run in triplicate. For both viruses, the PCR cycling consisted of 10 min at 95°C, followed by 40 cycles of 95°C for 10 s, and 60°C for 45 s. Data acquisition and analysis were carried out with Rotor Gene real-time analysis software 6.0 (Corbett Robotics, Brisbane, Australia). Serial dilutions from previously constructed HPLV and IHHNV plasmids were used to determine a standard linear relationship.

5.3 RESULTS

5.3.1 Cell Culture

No CPE was observed in any of the hybrid cell lines inoculated with either virus. Three flasks inoculated with HPLV (SF-9, F11-25, FHM) fell victim to fungal contamination on day nine, and were subsequently discarded. The flask with EPC cells was discarded on day 12, due to fungal contamination. The remaining cells showed normal fibroblastic structure throughout the 14 d period of exposure (Figure 5.1). The control flasks also remained confluent, with no evidence of cell detachment or lysis.

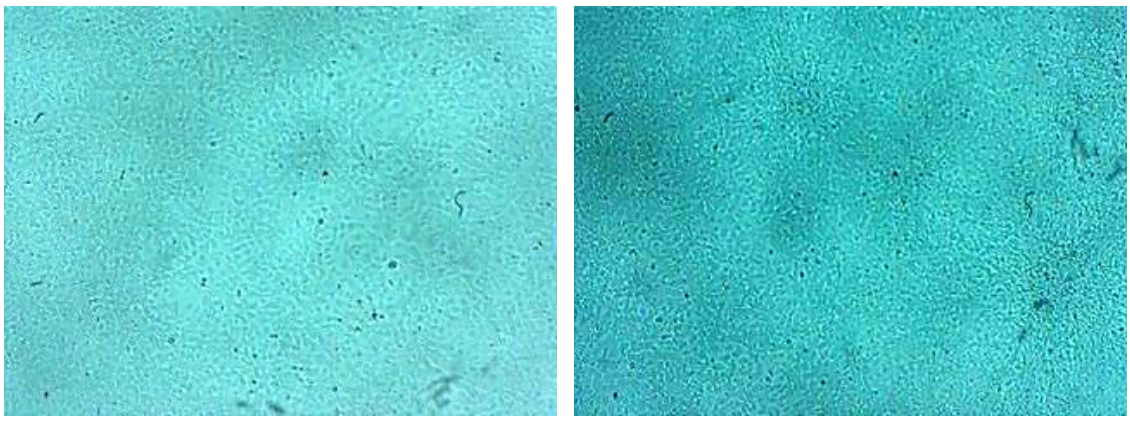
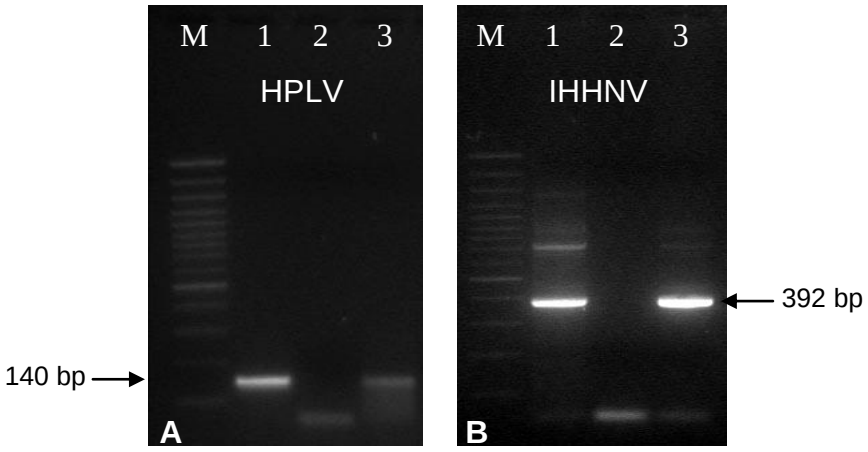


Figure 5.1. Hybrid cell line F14 inoculated with HPLV at (A) day 3 and (B) day 14.

5.3.2 Verification of Virus

The presence of viral DNA was confirmed using conventional PCR (Figure 5.2). Both PCR experiments contained template DNA extracted from purified virus, along with a negative and positive control.



Figures 5.2. Results of viral purification PCR showing bands at the expected amplicon size of (A) 140 bp for HPLV and (B) 392bp for IHHNV. Lane M: GeneRuler; Lane 1: Purified virus; Lane 2: Negative control; Lane 3: Positive control.

5.3.3 Viral Quantification

Approximately 8.26 x 10^3 viral copies/µl were detected at day 0 in the cell culture flasks exposed to HPLV. This level slowly decreased or remained stable during the 14 d experimental period (Table 5.1).

Table 5.1. Results of real-time PCR using hybrid cell lines inoculated with HPLV.

	Cell Line	Time (Days)	Average Viral Copies (per µl)	Virus Replication
SF-9 HYBRID	F1125	0	2.33E+05	
		3	6.52E+05	
		7	1.14E+05	Stable
	SF9	0	1.14E+04	
		3	1.87E+04	
		7	2.30E+01	Decrease
EPC HYBRID	F12C1	0	2.89E+06	
		3	2.69E+05	
		7	1.84E+05	
		10	6.10E+04	
		14	3.68E+04	Decrease
	F13B8	0	1.16E+03	
		3	1.89E+03	
		7	6.75E+03	
		10	3.69E+01	
		14	1.74E+03	Stable
	F17C7	0	3.72E+03	
		3	6.61E+03	
		7	1.61E+02	
		10	1.47E+04	
		14	1.50E+04	Stable
	EPC	0	1.32E+04	
		3	7.63E+04	
		7	3.10E+05	
		10	1.25E+05	Stable
FHM HYBRID	F14C1	0	1.49E+05	
		3	1.90E+05	
		7	1.96E+05	
		10	1.44E+05	
		14	7.95E+04	Stable
	F15D10	0	1.26E+03	
		3	3.32E+04	
		7	1.62E+04	
		10	9.59E+02	
		14	3.07E+02	Stable
	F16F6	0	2.73E+03	
		3	5.87E+03	
		7	1.93E+04	
		10	2.14E+04	
		14	9.51E+03	Stable
	FHM	0	8.29E+04	
		3	5.49E+04	
		7	1.43E+05	Stable

Approximately 9.77 x 10^3 viral copies/µl were detected at day 0 in the cell culture flasks exposed to IHHNV. This level slowly decreased for all cell lines during the 14 d experimental period (Table 5.2).

Table 5.2. Results of real-time PCR using hybrid cell lines inoculated with IHHNV.

	Cell Line	Time (Days)	Average Viral Copies (per µl)	Virus Replication
SF9 HYBRID	F1129	0	2.12E+03	
		3	1.62E+03	
		7	8.56E+02	
		10	4.80E+02	
		14	4.01E+02	Decrease
	SF9	0	1.53E+04	
		3	1.91E+03	
		7	3.66E+02	
		10	2.78E+02	
		14	0.00E+00	Decrease
EPC HYBRID	F12E3	0	1.75E+04	
		3	7.01E+03	
		7	7.13E+02	
		10	3.06E+02	
		14	1.36E+02	Decrease
	F13C12	0	1.59E+04	
		3	1.71E+03	
		7	9.11E+02	
		10	4.40E+02	
		14	1.67E+02	Decrease
	F17C7	0	4.24E+03	
		3	1.34E+03	
		7	1.92E+02	
		10	2.69E+02	
		14	8.60E+01	Decrease
	EPC	0	2.53E+04	
		3	9.39E+03	
		7	6.79E+02	
		10	6.26E+02	
		14	2.63E+02	Decrease
FHM HYBRID	F14C2	0	1.96E+04	
		3	1.76E+03	
		7	5.84E+02	
		10	3.55E+02	
		14	2.32E+02	Decrease
	F15D11	0	2.00E+03	
		3	6.66E+02	
		7	2.67E+02	
		10	1.04E+02	
		14	1.80E+01	Decrease
	F16F6	0	9.06E+02	
		3	2.91E+02	
		7	1.48E+02	
		10	1.26E+02	
		14	0.00E+00	Decrease
	FHM	0	4.65E+03	
		3	5.62E+02	
		7	1.26E+02	
		10	5.10E+01	
		14	2.10E+02	Decrease

Results of the real-time PCR showed that the number of viral genome copies in the cell culture media did not increase for any of the hybrid or control cell lines challenged with either parvovirus. This suggests that no viral replication occurred, but that some residual virus remained in the inoculum. While small changes were observed in the estimated number of viral copies during the experiment, to accommodate these small differences, only if the number of viral copies present on day 14 was approximately one log or more lower than the number on day 0 (ie. > 10% difference) were viral copies considered to decrease (Figure 5.3A). If the number on day 14 was one log or more greater than the number on day 0 (ie. > 10% difference), viral copies were considered to increase. A stable value was reported if there was less than one log change in the number of viral copies from the start to the end of the experiment (Figure 5.3B).

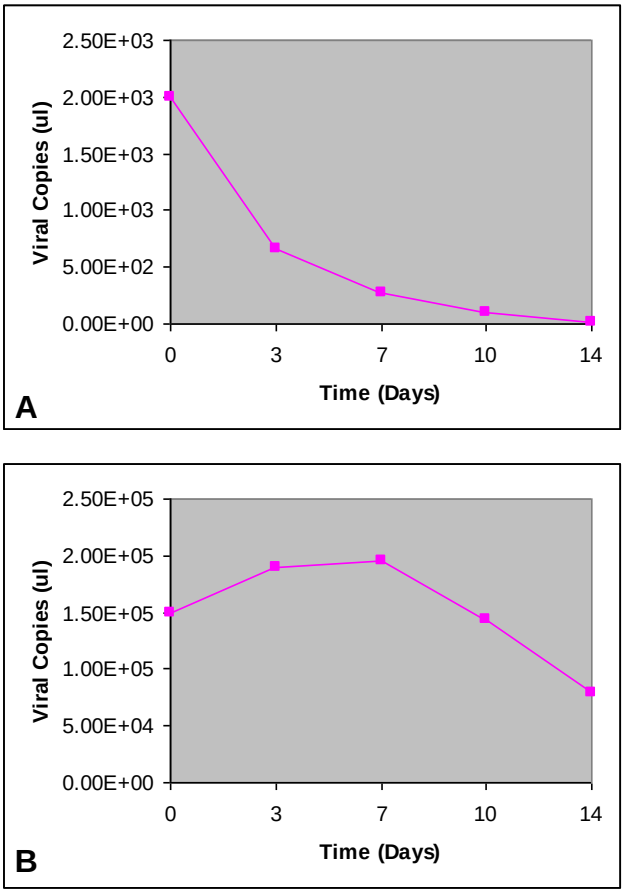


Figure 5.3. Viral load in two hybrid cell lines over the 14 d experimental period. (A) Cell hybrid F15-D11 exhibited a typical decrease in IHHNV virus copies over time. (B) Cell hybrid F14-C1A exhibited a stable HPLV load over time.

5.4 DISCUSSION

In an effort to develop a standardised cell culture system capable of supporting crustacean viral replication, previously developed crustacean hybrid cell lines were assessed for their ability to support viral growth. Two parvoviruses were successfully purified and isolated from penaeid tissue, and their presence was confirmed by PCR before inoculation into ten cell lines. Real-time PCR was used to assess viral loading over an experimental period of 14 days. Neither hybrid nor control cell lines challenged with either virus exhibited an increase in viral load, suggesting a lack of viral replication, nor did any of the cell lines display CPE from viral infection.

If viral replication was indeed absent, each flask challenged with virus should have shown a small dilution effect over time, due to the 1 ml of culture medium removed and replaced with fresh medium at each time point. This was the case with cultures inoculated with IHHNV, in which all cell lines decreased in viral titre throughout the 14 days. However, seven of the cell lines challenged with HPLV had viral loads that remained relatively stable. Although this result suggests a low level of viral replication, the absence of CPE during the experiment, along with subsequent blind passages at the end of the experiment (data not presented), confirmed a lack of viral replication. It is possible that the aliquots of culture medium taken at each time point were not uniform. Alternatively, HPLV may stay viable in cell culture medium longer than IHHNV. A protective effect of FBS in the culture medium (Johnson, 1990; Jensen and Johnson, 1994; Dunn *et al.*, 2003; Pantoja *et al.*, 2004) may also have contributed to the stability of the viral loads. This protective effect of FBS on viruses has been documented previously (Johnson, 1990; Jensen and Johnson, 1994; Dunn *et al.*, 2003), and research has found that the best viral transport media for the preservation of viral viability are those that contain FBS (Pantoja *et al.*, 2004).

The absence of viral replication in the hybrid cell populations may have been due to a lack of appropriate cell surface receptors by both parvoviruses investigated. Viral infection is a complicated pathological process that can be affected by many factors, with receptor binding and penetration considered

basic requirements (Jiang *et al.*, 2005). Other methods have proven to be successful in introducing foreign DNA into cells *in vitro*, including lipofectamine (He *et al.*, 2006), calcium-phosphate precipitation (Jordan *et al.*, 1996), microinjection, and electroporation (Sun *et al.*, 2005). These delivery methods may provide a more efficient approach for viral entry into the cell.

Susceptibility of the *C. quadricarinatus* hybrids to parvovirus infections is also uncertain, as this species has never supported natural infections of either virus. However, *P. monodon* are known to be a natural host for both viruses, and therefore cell hybrid partners were more likely candidates to support viral replication in this study.

Quantitative real-time PCR provides an accurate method of detecting the presence and replication of viruses in cell culture (Pantoja *et al.*, 2004). Cell culture tends to be a traditional discipline with simple protocols, and has been slow to adopt molecular methods that could accelerate its development. Most previous studies detected viruses in cell culture by the presence of CPE, using endpoint dilutions to quantify the number of viruses in fish cell lines (Woodland *et al.*, 2002), or quantifying the number of plaques formed in culture to estimate the degree of CPE (McClenahan *et al.*, 2005). Whilst traditional PCR can be used to detect the presence of viral DNA in cell cultures (Assavalapsakul et al., 2003; Jiravanichpaisal *et al*., 2007), its use is very limited in quantitating viral loads in cell cultures. The present study demonstrates that recent technological advances, such as real-time PCR, can be successfully applied to viral investigations in cell culture studies.

The lack of a continuous cell culture system has limited virological studies in crustaceans. A standardised permanent culture system would allow amplification of crustacean viruses; however, primary cell cultures can be utilised until a permanent cell culture model becomes available. To date, primary cell cultures have allowed investigations into crustacean viruses, such as WSSV, YHV, and PRBV. However, there is no report to date of a parvovirus being successfully propagated in a crustacean primary cell culture. Unfortunately, the observed inability of the developed hybrid cells to support

crustacean viral replication, as well as the demonstrated absence of crustacean genes and proteins in the hybrid cells (Chapter 4), suggest that these hybrid cell populations lack key crustacean cell components required for their use as a crustacean *in vitro* system.

CHAPTER SIX
OPTIMISATION OF PRIMARY CELL CULTURE METHODS FOR *CHERAX QUADRICARINATUS*

6.1 INTRODUCTION

In the past decade, freshwater crayfish aquaculture has expanded rapidly, both in Australia and around the world. Australia has one of the richest endemic faunas of freshwater crayfish, and *Cherax quadricarinatus* is the most popular cultured species, with annual aquaculture production of 100 *t* (ABARE, 2008). Disease problems constitute the largest single cause of economic losses in aquaculture (Meyer, 1991) and continue to limit the productivity of this industry. To date, nine viruses have been identified that infect freshwater crayfish (Edgerton *et al.*, 2000; Edgerton *et al.*, 2002). Of the viruses found only in freshwater crayfish, none have been purified sufficiently to permit further characterisation beyond preliminary descriptions of their morphology, pathology and epizootiology (Evans and Edgerton, 2001). The limited knowledge of these viruses is attributable to a lack of sensitive diagnostic methods for viral detection and study, such as the establishment of a crustacean cell line for *in vitro* viral replication and analysis.

The most widely studied disease organism of freshwater crayfish is an intranuclear bacilliform virus (IBV). The IBV of *C. quadricarinatus* infects the hepatopancreas and has caused mortalities in farms worldwide (Edgerton and Owens, 1993; Groff *et al.*, 1993; Edgerton *et al.*, 1995; Hedrick *et al.*, 1995; Edgerton and Owens, 1997; Jimenez and Romero, 1998; Romero and Jimenez, 2002). Like all of the viruses that infect *C. quadricarinatus*, no descriptive physiochemical characteristics are known, and the structure and function of the viral genome remain to be determined. The development of a cell culture system for *C. quadricarinatus* would be a significant step forward in the study of IBV and other viral diseases of *C. quadricarinatus*.

Although there have been many attempts to establish cell lines from crustaceans, few have attempted to establish cell lines from freshwater species. Once techniques have been established for the development of a cell line for

one crustacean species, this technology could possibly be transferred to initiate cell lines from other species. A healthy disease-free primary culture is required for the creation of a cell line, and spontaneous transformation of cells from primary cultures, although rare, has occurred in some insect cell cultures (Lynn, 1999). However, even induced transformation through the use of oncogenes or chemical mutagenesis needs a healthy proliferating culture of cells. The goal of the research described in Chapter 6 was to develop and optimise methods for establishing primary cell cultures from putative disease-free *C. quadricarinatus*. The variables examined included animal age, tissue selection, cell culture media, cell culture equipment, and procedures for culture initiation.

6.2 MATERIALS AND METHODS

6.2.1 Putative Virus-free *C. quadricarinatus*

The experimental population of *C. quadricarinatus* came from James Cook University, Townsville. As disease-free animals are required for primary cell culture, a population of presumptive virus-free *C. quadricarinatus* were used to establish the primary cell cultures. This population was produced by removing eggs from female brooders at the eyed stage, sterilising and hatching the eggs, and culturing the juveniles in uncontaminated water (Edgerton and Owens, 1997). Following their initial culture, the crayfish were maintained in an isolated biosecure area. To increase the virus-free population, a larger recirculating system was constructed, with six interconnected 1 t Reln© tanks and a header tank dedicated as the biofilter for the system (Figure 6.1).

Figure 6.1. Putative virus-free *C. quadricarinatus* were contained in six interconnecting tanks with recirculating water and biological filtration.

6.2.2 Tissue Comparison

Crayfish of various ages were targeted for cell culture, including embryos (eyed stage), young juveniles (up to 1 month old), and adolescent animals (~5-8 cm long). Whole bodies from the embryos and young juveniles were used to initiate cultures, while selected cell types were extracted from adolescent crayfish including gills, hepatopancreas, gonads, haemocytes and haematopoietic tissue.

6.2.3 Cell Culture Procedure

Crayfish were anesthetised by immersion in ice water for 5 min and then transferred to 2.5% formaldehyde for 10 min to rid them of external contamination. Animals were further surface-sterilised with 70% ethanol, and briefly washed in sterile deionised water before whole bodies or selected tissues were dissected into small pieces (~1 mm), using crossed scalpel blades in a laminar flow hood. Tissue fragments were either manually homogenised in 1.5 ml tubes or enzymatically dissociated in antibiotic holding medium, consisting of L-15 culture medium with 4× antibiotic mix (200 IU/ml penicillin, 200 µg/ml streptomycin, 80 µg/ml kanamycin, 5 µg/ml polymyxin B, and 2.5 µg/ml fungizone®) and 2.2 U/ml dispase. Dissociation was carried out for 30-45 min with a magnetic stirrer and three fleas. Homogenized or dissociated cells were pelleted by gentle centrifugation (500 × *g* for 5 min), using an Eppendorf

A-4-44 rotor. Three additional wash and centrifugation steps were performed with the antibiotic medium. Cultures were either directly seeded in culture vessels or passed through a 40 µm cell strainer (BD Falcon, North Ryde, Australia) to further remove excess debris. Cells were seeded into culture plates or flasks with one of three culture media. All cultures were incubated at 28°C. The culture medium was replaced after 24 h with an equal volume of fresh medium, followed by weekly medium changes.

Cultured cells were observed daily with an Olympus IMT-2 inverted phase-contrast microscope and images captured (Section 3.6). Viable cells counts were conducting according to the procedure described in Section 3.7.

6.2.4 Cell Culture Media

Cell viability and growth was compared using the three most common media used for culturing crustacean cells: L-15, double-strength L-15, and M-199. Media were reconstituted from powder containing L-glutamine (Gibco, Brisbane, Australia) by adding sterile deionised water and 2× antibiotic mix (200 IU/ml penicillin, 200 µg/ml streptomycin, 80 µg/ml kanamycin, 5 µg/ml polymyxin B), and filtered through a 0.22 µm filter. Following filtration, fungizone® (amphotericin B) was added to the media at a final concentration of 2.5µg/ml. Foetal bovine serum was added fresh to each medium at a concentration of 10%.

The ability of the three culture media to support *in vitro* proliferation of *C. quadricarinatus* cells was assessed using a cell proliferation kit II (Roche Applied Sciences, Brisbane, Australia), which measured the reduction of a yellow tetrazolium salt (XTT) to an orange formazan dye by metabolically active cells. One hundred µl of each culture medium was added individually to 16 wells of a 96-well plate; half of the wells were seeded with ~3×10^3 cells, and the other wells were used as medium-only controls. XTT mixture was prepared by mixing 2.5 ml of the XTT labelling reagent with 50 µl of the supplied electron coupling reagent. Fifty µl of XTT mixture was added to each well (final XTT concentration = 0.3 mg/ml), and dual absorbance readings (492nm and 650nm) were measured daily for 14 d, using a Labsystems multiskan EX plate reader.

Cell proliferation in each of the three media was estimated by subtracting the average value of A_{492}-A_{650} for the media-only wells from the average value for the wells seeded with cells.

The osmolality of each of the three media was measured and compared to that of crayfish haemolymph. Five hundred µl of haemolymph was extracted from each crayfish (n=10), using a 25 gauge needle inserted into the dorsal thoracic chamber. The extracted haemolymph was mixed with 50% Alsever's anticoagulant (Appendix 2) to avoid clotting. Osmolality of the haemolymph and media was measured using a Gonotec osmomat 030 cryoscopic osmometer (Berlin, Germany).

6.2.5 Cell Culture Equipment

A variety of cell culture containers were assessed for their ability to support cell attachment and growth: cell culture-treated 25 cm^2 flasks (Corning, Nunc, Sarstedt, Iwaki); non-treated 25 cm^2 flasks (Iwaki); 2 ml tubes (Nunc); 6, 12, 24, and 96-well plates (Nunc, Iwaki, TPP, Sarstedt).

Although, results of previous research suggested that crustacean cell replication is more prevalent when cells are attached to a substrate (Hsu *et al.*, 1995; Mulford *et al.*, 2001), some invertebrate cells, prefer to float and grow in suspension (Odintsova *et al.*, 2005). To examine replication of the crayfish cells in suspension, a floating substrate (microcarrier) was added to some cultures. This microcarrier, Cytodex®1 (Sigma, Brisbane, Australia), was composed of a cross-linked dextran matrix with positively charged N,N-diethylaminoethyl (DEAE) groups.

To encourage cell attachment to some of the culture vessels, the flasks and wells were coated with two different attachment factors. The first was a 2% gelatin solution (Sigma-Aldrich, Brisbane, Australia), which was applied to the substrate (10 µl/cm^2) and allowed to dry on the surface for at least 2 h before the introduction of medium and cells. The second attachment factor was extracted from crayfish muscle tissue. Briefly, 1g of muscle tissue was homogenised in 1 ml of PBS, the homogenate was centrifuged at 5,000 x *g* for

30 min at 4°C, and the supernatant was filtered through 0.45 µm and 0.22 µm membranes. Ten µl/cm^2 were applied to the substrate and left undisturbed for 2 h prior to the introduction of medium and cells.

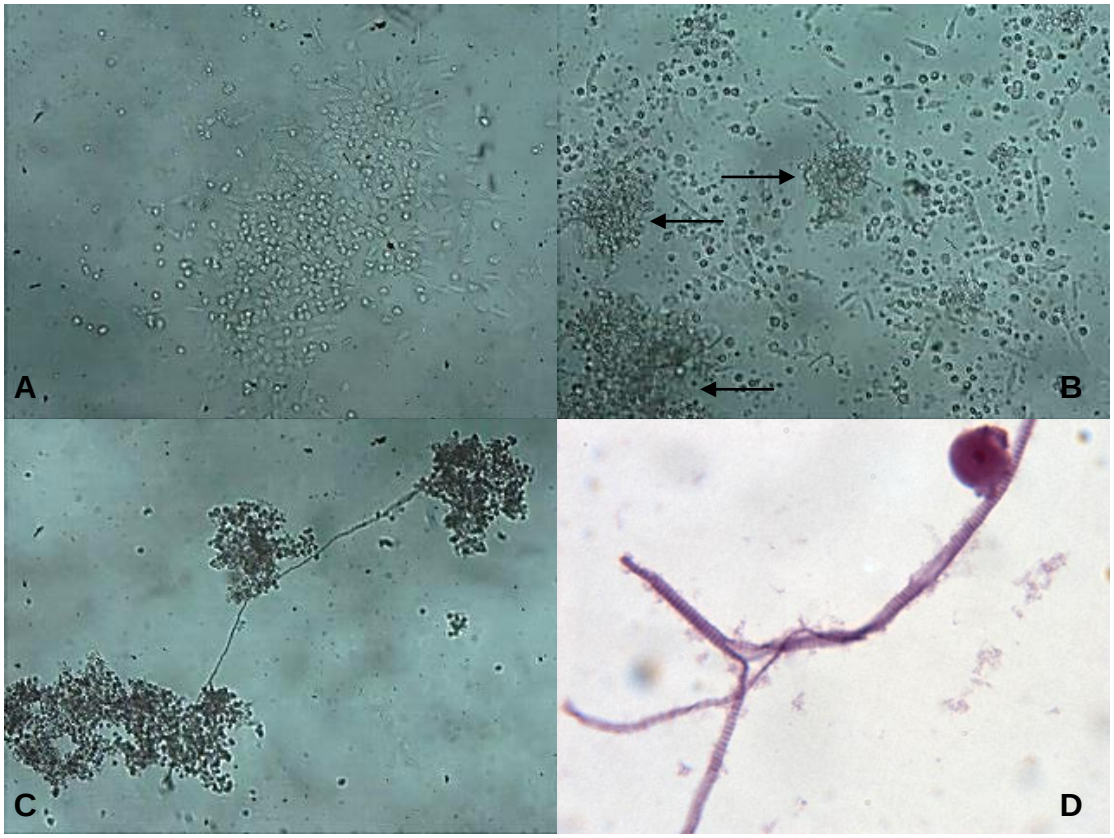
6.3 RESULTS

6.3.1 Tissue Comparison

Viable primary cultures were achieved with all tissues extracted from *C. quadricarinatus* (Table 6.1). Haemocyte cultures contained the most attached cells (Figure 6.2A); however, no replication of these cells was observed. Cultures of embryonic and gill tissues contained a lot of floating debris, with only a few cells attached to the substrate. Hepatopancreas and gonadal cultures showed very little replication and remained viable for less than one week. Haematopoietic tissue was found to be the best tissue source. A high percentage of haematopoietic cells attached to the culture vessels, underwent some replication, and remained viable for more than 4 months (Figure 6.2B). As with all cells, replication was highest during the first 24 h, some replication occurred for 72 h, and then most cells remained viable, but continued to float in suspension in a non-replicative state. In some cultures, a cellular matrix formed, composed of long striated muscle-like spindles, in which the cells would attach and grow (Figure 6.2C,D).

Table 6.1. Characteristics of *C. quadricarinatus* cultured cells from different tissues.

Tissue/Cell Type	Cell Attachment	Replication	Viability	Longetivity
Haematopoietic Cells	Attached to culture flask	> 72 hrs	High	> 4 months
Haemocytes	Attached to culture flask	Nil	High	1 week
Embryonic Cells	Mainly suspension cultures; semi-attached	72 hrs	High	3 weeks
Cells from Gill Tissue	Mainly suspension cultures	24 hrs	Low	< 1 week
Hepatopancreatic Cells	Mainly suspension cultures	24 hrs	Low	< 1week
Cells from Gonadal Tissue	Mainly suspension cultures	24 hrs	Low	<1 week

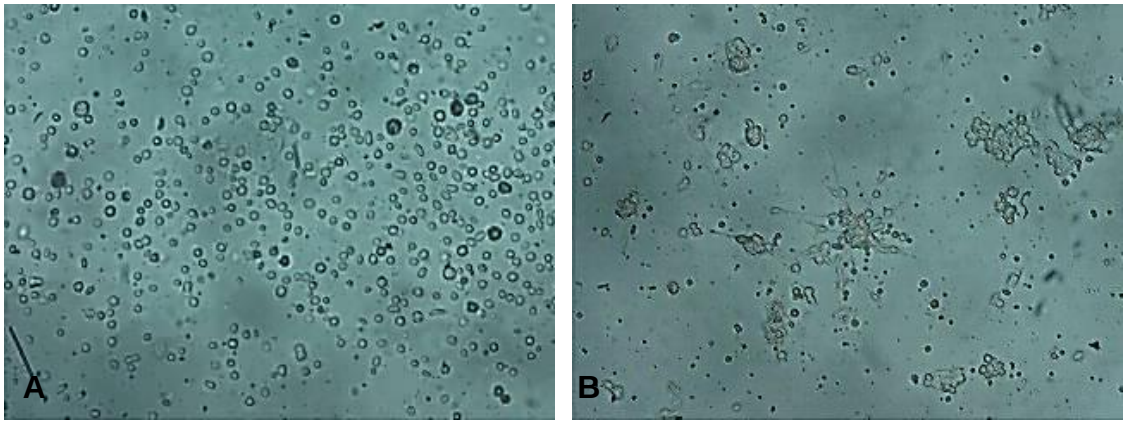


Figures 6.2. Photomicrographs of cultured cells from different tissues. (A) Haemocytes attached rapidly to culture substrates. (B) Haematopoietic tissue cultures contained many cells that attached, and some formed small aggregates (arrows). (C and D) Embryonic cell cultures primarily contained suspended cells that formed 3-dimensional cell matrices containing long striated spindles. (D) Striated spindles stained with haematoxylin and eosin.

6.3.2 Cell Culture Procedure

Centrifugation did not efficiently eliminate all debris. Passing the cell suspension through a 40 µm cell strainer provided a more efficient method and allowed for better visualisation of the cells.

Enzymatic disassociation of the cells was more effective than mechanical homogenisation. Cell cultures that were enzymatically treated produced a higher number of single cells, and a high proportion of these attached to the substrate. Aggregates of cells from the mechanical disassociation floated in suspension and did not appear to replicate as frequently (Figure 6.3).



Figures 6.3. (A) Cultures of enzymatically disassociated cells exhibited more single cells than (B) mechanically disassociated cells, which had many small aggregates of tissues and cells.

Medium changes proved to be troublesome. Because most of the cells (viable and non-viable) were suspended, medium changes involved removing the cells from the medium by centrifuging, resuspending the cells in fresh medium, and seeding the cells into new culture containers. The centrifugation caused any cellular matrix that had formed to break up. The matrix often re-established itself at decreased density after one week. Therefore, medium changes were performed monthly, rather than weekly.

6.3.3 Cell Culture Media

Cell proliferation was much higher in single strength L-15 culture medium than in double-strength L-15 (Figure 6.4). M-199 culture medium gave the lowest proliferation rates. The L-15 medium was further optimised for crustacean cells in later experiments (Chapter 7).

The osmolality of crayfish haemolymph was higher than the osmolality of single-strength L-15 and M199 culture media, with or without FBS, but lower than that of the double-strength L-15 (Table 6.2). Single-strength L-15 medium with FBS, supplemented with 10% (v/v) 1M NaCl, matched the osmolality of crayfish haemolymph.

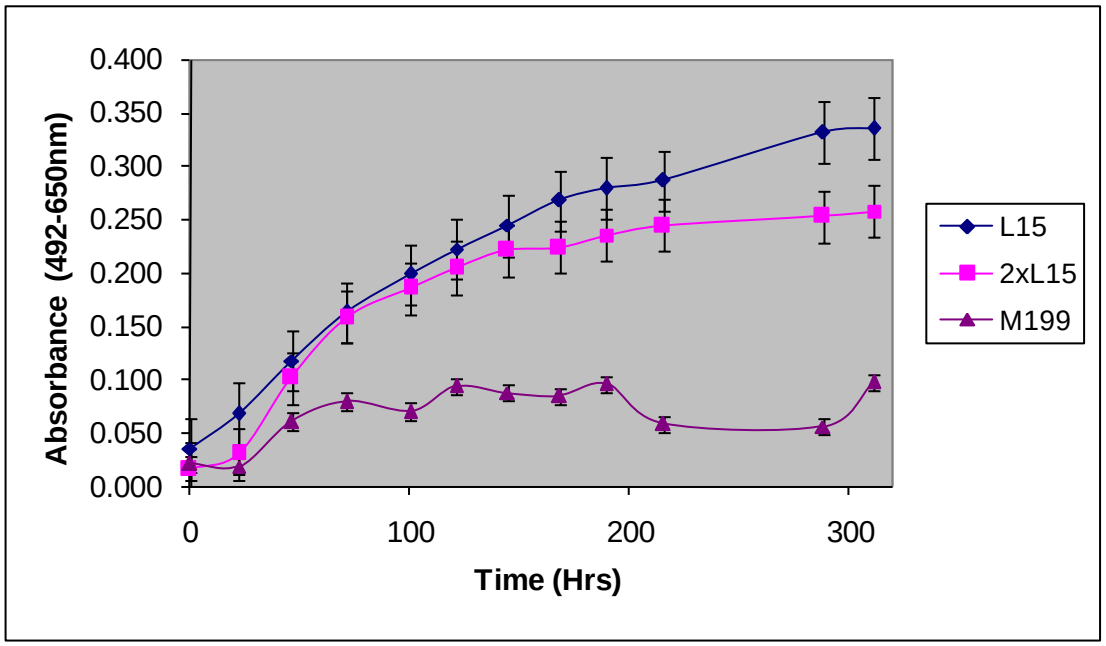


Figure 6.4. Comparison of cell proliferation in three culture media, based on the XTT assay. All media were supplemented with 10% FBS.

Table 6.2. Osmolality of culture media, crustacean haemolymph, and L-15 medium supplemented with sodium chloride.

SAMPLE	OSMOLALITY RANGE (mOsm/kg)	AVERAGE (mOsm/kg)
L-15	285	**285**
L-15 +10% FBS	286-304	**295**
2×L-15	582	**582**
2×L-15 +10% FBS	615	**615**
M199	257	**257**
M199 +10% FBS	274	**274**
Alsever's (Anti-coagulant)	353-361	**357**
Crayfish Haemolymph (with 50% Alsever's)	385-410	**396**
Adjusted Crayfish Haemolymph*	413-463	**435**
L-15 +10% FBS + 10% NaCl (1M)	434	**434**

*Adjusted osmolality of crayfish haemolymph was calculated from the measured osmolality of haemolymph + Alsever's anticoagulant, using the equation 0.5(x) + 0.5(y) = 396, where y is the osmolality of Alsever's anticoagulant, and x is the unknown osmolality of haemolymph.

6.3.4 Cell Culture Equipment

Cell growth and attachment showed no differences when different brands of cell culture equipment were used. Interestingly, there was no difference in attachment rate between culture-treated and non-treated flasks. Pre-treatment with attachment factors did not increase the level of cellular attachment. Conversely, coating the surfaces with gelatin caused a large decrease in the number of attached and viable cells in culture. The use of cytodex-1 microcarriers also failed to enhance cell proliferation in the suspension cultures. Some cells did attach to the microcarriers (Figure 6.5), but no increase in replication was observed compared to cultures without microcarriers.

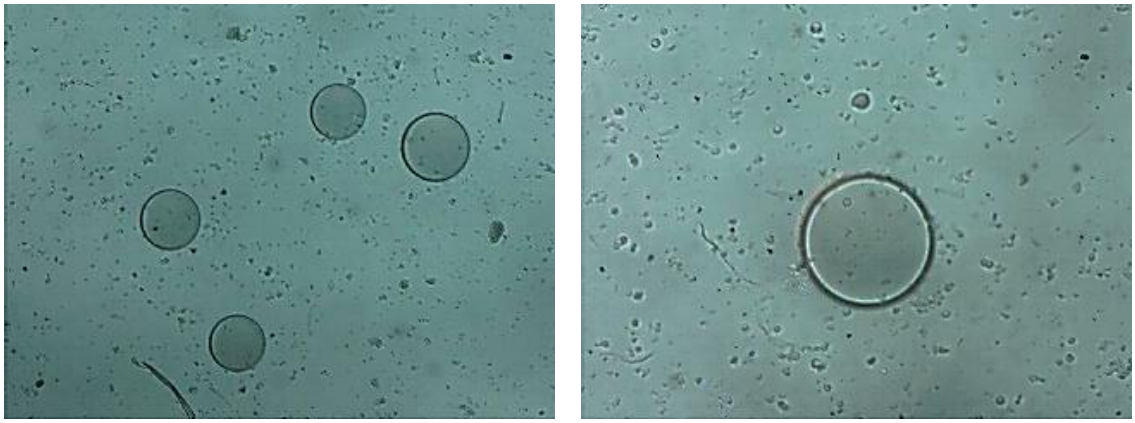


Figure 6.5. Primary culture of *C. quadricarinatus* cells initiated with cytodex-1 microcarrier.

6.4 DISCUSSION

6.4.1 Tissue Source

Key factors in development of *in vitro* cells cultures include selecting an abundantly available species, considering its suitability for experimentation, and choosing an appropriate tissue source. The best tissue for crustacean cell culture is controversial (Mothersill and Austin, 2000), and disagreements might be attributable to the previous use of different species and media, making it difficult to compare results. During the last 20 years, studies of crustacean cell culture have used 18 species, 17 tissues, and 26 different culture media (Appendix 3). Regardless of the tissue type used for culture initiation in previous studies, many of the primary cell cultures only grew in suspension,

while others grew as a multi-layered mass that occasionally was loosely attached to the surface of the culture vessel (Odintsova *et al.*, 2005).

Results of the present study indicated that initiation of primary cell cultures from *C. quadricarinatus* was most successful using haematopoietic tissue. Of the tissues tested, these cell cultures remained viable for the longest period and also contained the highest number of proliferating cells. Haematopoietic cells may also have the greatest potential to form immortal cell lines through spontaneous transformation, due to their longevity in culture. Theoretically, any cell type can be cultured, but the survival and *in vitro* use of the cells depend on the degree of differentiation. Aquatic invertebrates, such as crayfish, must possess tissues with pluripotent and totipotent cells, based on their considerable capacity for tissue regeneration and cell replacement (Odintsova *et al.*, 2005). Cultures initiated from gonads, hepatopancreas, and haemocytes contain many cells that are mature and differentiated. Cells from these tissues are therefore considered to have a finite lifespan and are likely to produce finite cultures (Mothersill and Austin, 2000). In contrast, previous research concluded that the haematopoietic tissue of at least one crayfish, *Pacifastacus leniusculus,* is a source of cells with a potentially infinite lifespan (Söderhall *et al.*, 2003). Thus, haematopoietic cells appear to be a promising source, and the present study demonstrated that haematopoietic cells from *C. quadricarinatus* could be maintained as viable primary cell cultures. However, the low mitotic activity exhibited by these cultures might be a major impediment to the development of a cell line. This lack of mitotic ability has also been observed in other marine invertebrate cell cultures (Grace, 1965; Derikot and Blinova, 1982; Kawamura and Fujiwara, 1995; Odintsova *et al.*, 2005). Even if the haematopoietic cell cultures of *C. quadricarinatus* had shown high rates of mitosis, their ability to support viral replication has yet to be determined.

6.4.2 Culture procedures and media

The method used to initiate cell cultures can influence the survival of the cells. Results of the present study indicated that enzymatic cell dissociation of *C. quadricarinatus* tissues by dispase was more successful than mechanical dissociation in obtaining proliferating semi-attached cells. Although mechanical

dissociation is suitable for loose tissues (Toullec, 1999), this method seems to reduce the ability of cells to attach to the substrate. Fragile cell types are often broken, and many cells are ruptured during mechanical treatment, which releases proteases in the medium and can lead to cell degradation (Freshney, 2005). Enzymatic dissociation is less disruptive than mechanical dissociation, as the connective tissue is digested by the enzyme, and viable cells are separated (Toullec, 1999).

Enzymatic cell dissociation using dispase proved suitable for initiating cell cultures of *C. quadricarinatus* tissue. Dispase is a neutral protease, isolated from culture filtrates of *Bacillus polymyxa* (Stenn *et al.*, 1989), compared to trypsin which is serine protease. Dispase is a relatively weak and gentle enzyme for tissue dissociation, and appears to cause less damage to cells, especially to those that are sensitive to trypsination. Enzymatic dissociation is particularly useful for dissociation of compact tissues, but can also be applied to loose tissues. However, results of previous research showed that, although enzymatically dissociated cultures of crustacean cells survived well, their rate of cell multiplication was low (Mothersill and Austin, 2000). When sub-cultured, furthermore, there was no cell proliferation and little success beyond the primary cell culture stage was obtained (Mothersill and Austin, 2000). Therefore, the successful initiation of *C. quadricarinatus* cells cultures using dispase does not necessarily mean that these cells will proliferate better.

M199 and L-15 media have consistently produced successful primary cultures from a variety of crustacean tissues. These two media differ significantly in composition and concentrations of components (Lang *et al.*, 2002a). Both M199 and L-15 are commonly used to maintain fish cell cultures, but L-15 is used more frequently (Lee *et al.*, 1993), and is reported to be more successful than M199 in initiating primary fish cell cultures (Schreera *et al.*, 2005). The success of L-15 may be attributable to its greater buffering capacity, which allows cells to utilise free amino acids and to substitute galactose and pyruvate for glucose, ultimately increasing their endogenous production of CO_2 (Griffiths, 1986). While M199 contains a wider variety of vitamins and amino acids, the vitamins and amino acids included in L-15 are at higher concentrations. Thus,

higher concentrations of key amino acids and vitamins may account for the greater ability of *C. quadricarinatus* cells to proliferate in standard L-15 than in M199. Conversely, the much higher concentrations in double strength L-15 may have been too potent. Until further information about the specific nutritional requirements of *C. quadricarinatus* cells is available, the reason for their differential performance in different media remains speculative.

The osmolality of L-15 medium must be adjusted to match the osmolality of crayfish haemolymph. The osmolality determined for *C. quadricarinatus* haemolymph (435 mOsm/kg) was within the range reported for other freshwater crayfish (340-450 mOsm/kg, Mothersill and Austin, 2000). Most of the defined cell culture media, including L-15 and M199, are adjusted to the osmolality of vertebrate blood (230-280 mOsm/kg). Therefore, matching the osmolality according to the species used in cell culture appears appropriate.

6.4.3 Culture Equipment

Cell-cell adhesion and cell-substrate adhesion are critical for the development and functioning of multicellular organisms (Johansson, 1999). However, there have been few specific studies of adhesion factors in crustacean cells. Frerichs (1996) found that cell proliferation of embryonic *Macrobrachium rosenbergii* occurred most noticeably in tissues/cells adhering to a flask or plate surface. Failure of cultured cells to attach to the vessel surface may be the most important single factor preventing success of cell line development.

Cell attachment is influenced by properties of the available surface, i.e. the culture container. The commercially available culture flasks are composed of polystyrene, which is hydrophobic and may not provide a suitable surface for cell attachment. Therefore, polystyrene culture vessels are treated using corona discharge, gas plasma, γ-irradiation, or chemicals to produce a charged, wettable surface (Freshney, 2005). Nevertheless, proliferation and attachment of *C. quadricarinatus* cells showed no notable differences between four brands of culture flasks. Even pre-treated culture vessels had no noticeable effect. Gelatin and crayfish muscle extract to coat the culture substrate before cell introductions, were also investigated. However, these two heterogeneous

protein mixes did not have any positive effects on cell attachment. Conversely, the gelatin-coated flasks caused rapid deterioration of cell cultures. This may have been due to an adverse reaction to some of the proteins in gelatin, which is derived from mammalian skin, tendons, ligaments and bones (Lee *et al.*, 1992) and, therefore, may be more suitable for enhancing attachment of mammalian cells.

Although lack of attachment to a substrate may be a limiting factor for cell proliferation, detachment from the culture vessels has been observed in cell cultures from a number of aquatic invertebrates, including sea cucumbers (Odintsova *et al.*, 2005) and oysters (Mialhe *et al.*, 1988; Domart-Coulon *et al.*, 1994). Cell cultures from many insects also prefer to grow in suspension, rather than attached to a substrate (Lynn, 1999). *Cherax quadricarinatus* cells, like other arthropod and marine invertebrate cells, may preferentially grow in suspension. However, the addition of cytodex-1 microcarriers failed to enhance cell proliferation in suspension cultures, suggesting that this is not a promising approach.

6.4.4 Conclusions

The present study is the first to initiate and optimise methods for establishing cell cultures from the crayfish, *C. quadricarinatus*. Many of the established cultures contain healthy viable cells, which will continue to be incubated and observed for spontaneous transformation. These primary cell cultures can also be used for cell transfection studies with oncogenes in an attempt to expedite cell immortality.

CHAPTER SEVEN
CULTURE MEDIA OPTIMISATION

7.1 INTRODUCTION

The culture medium is the most important element in cell culture, significantly impacting on the survival of cells. The selection of a culture medium is generally based on the requirements of the organism. With aquatic animals, this involves consideration of the external environment (marine or freshwater), the internal environment (specific nutrient requirements of the animal), and carbon dioxide or buffering systems that might be used (Mothersill and Austin, 2000). Nothing is known about the precise nutritional requirements of crustacean cells, and therefore, the medium selection process is largely guesswork (Mothersill and Austin, 2000). The lack of information accounts for the low frequency of mitosis observed in aquatic invertebrate cell cultures to date (Mialhe *et al.*, 1988; Domart-Coulon *et al.*, 1994).

Many previous studies of crustacean cell culture initially used Grace's insect medium, due to the success of this medium in culturing other arthropod cells. To date, research in crustacean cell culture has investigated 25 other media; Leibovitz's (L-15) at single and double strength and medium 199 (M199) have proven to be most successful (Appendix 3). These commercial media were developed for vertebrate cell culture, and may not contain all of the *de novo* factors required for invertebrate cell growth. Two previous studies focused on biochemical analysis of crustacean haemolymph, and demonstrated that cultured cells performed better in optimised media, i.e. established monolayers and delayed onset of cellular senescence (Najafabadi *et al.,* 1992; Shimizu *et al.,* 2001). Even with optimised media, however, there was no evidence of sustained DNA or protein synthesis.

The present study tested the hypothesis that the proliferative capacity of crustacean cell cultures is not only limited by the amount of nutrients provided by the culture media, but also by the absence of specific nutrients or growth-promoting factors. The method commonly used to determine physiological requirements of cultured crustacean cells – and to design and modify cell

culture media - involves qualitative observations by light microscopy. In the present study, the effect of supplementation of common culture media was assessed by measuring cell proliferation, using a colourimetric assay consisting of tetrazolium salts. This simple test is based on the reduction of the yellow tetrazolium salt into an orange formazan dye by metabolically active cells. The formazan dye is soluble in aqueous solutions and can be directly quantified with an ELISA plate reader. The readings can be statistically analysed to determine which medium supports the largest number of metabolically active cells (referred to herein as cell proliferation). This method has been successfully used to measure proliferation in cell cultures of insects (Hatt *et al.*, 2001) and molluscs (Domart-Coulon *et al.,* 1994; Auzoux-Bordenave *et al.,* 2007). It has also been used to optimise media for mammalian cell cultures (Chun *et al.*, 2003). However, this technique has not been applied to crustacean cell cultures.

7.2 MATERIALS AND METHODS

7.2.1 Elemental Composition of Haemolymph

QuantiChrom kits (Bioassay Systems, Hayward, USA) were used to assess key chemical elements in the crayfish, *Cherax quadricarinatus,* and the prawn, *Penaeus monodon*. Four kits were used to measure phosphate, calcium, iron, and copper concentrations in the haemolymph of 10 individuals of each species. The results were compared with concentrations of the same elements in commonly used cell culture media (single and double strength L-15 and M199) supplemented 10% FBS.

For each sample, 200 µl of QuantiChrom working reagent was added to 50 µl of sample. After incubation at room temperature, absorbance was measured at 610, 540, 650, and 354 nm, respectively, for calcium, iron, phosphate and copper. A calibration curve for each element was determined with a series of standards, and the slope of each curve was calculated by linear regression analysis. For each sample result, the background was adjusted by subtracting absorbance of a well containing no sample, and the adjusted sample

absorbance was divided by the slope of the calibration curve to determine concentration.

7.2.2 Effects of Media Supplementation on Cell Proliferation

Cell proliferation was estimated based on the metabolic activity of mitochondrial dehydrogenases, using the WST-1 Assay (Roche Applied Science, Brisbane, Australia). Five concentrations of a specific supplement were compared simultaneously in one 96-well plate. Sixteen wells of the same supplement concentration were set up; half of these were seeded with cells and the other half were unseeded media controls (Figure 7.1). L-15 with adjusted osmolality was the basal medium (Section 6.3.3).

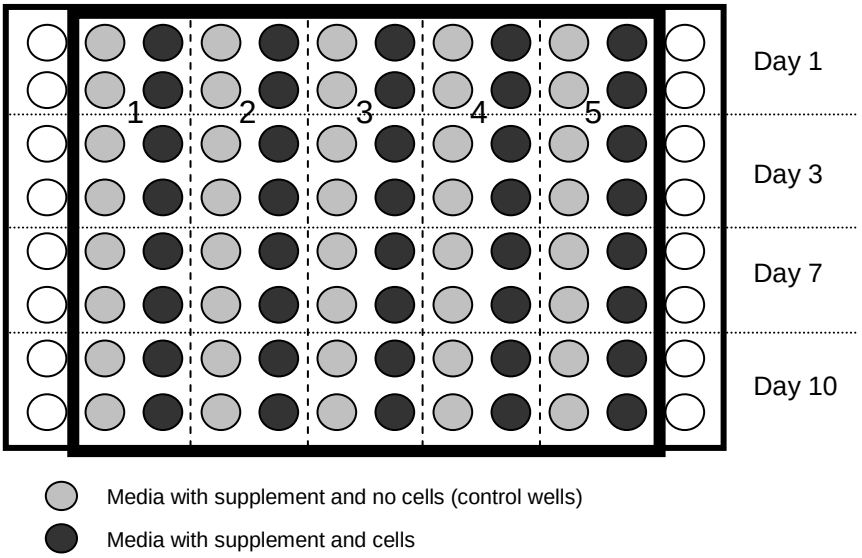


Figure 7.1. Plate layout used for WST-1 assay. Five concentrations of a culture medium supplement (columns 1-5) were assessed simultaneously at four different time points.

Primary cell cultures were initiated from 1-2 week old *C. quadricarinatus*, according to the procedure described in Section 6.2.3. Approximately 3×10^3 cells were seeded in each well in 100 µl of culture medium. Ten µl of the WST-1 reagent was added to duplicate wells on days 1, 3, 7, and 10, and absorbance was measured immediately at 450 nm with a reference wavelength of 650 nm, on a Labsystems multiskan EX plate reader. Cell proliferation for each supplement at each time point was estimated by calculating the average relative

absorbance ($A_{450} – A_{650}$) of the duplicate wells with cells, and subtracting the average relative absorbance of the duplicate wells with no cells. To allow a nonbiased analysis of results, statistical analysis was performed by univariate analysis of variance (ANOVA). Data was normally distributed as seen by Q-Q plots. When ANOVA tests showed significant differences, comparisons between individual means was performed using least significant difference (LSD).

A variety of supplements and concentration ranges were compared for their ability to support crustacean cell proliferation: 1-8 g/L glucose (Gibco, Brisbane, Australia), 0.1-4 g/L lactalbumin hydrolysate (Sigma, Brisbane, Australia), 0.1-4 g/L yeastolate (Sigma, Brisbane, Australia), 0.5-4% cholesterol (Gibco, Brisbane, Australia), 2.5-20% non-essential amino acids (Gibco, Brisbane, Australia), 1-4 mM glutaMAX™ (Gibco, Brisbane, Australia), 5-40 mM HEPES buffer (Sigma, Brisbane, Australia), 50-400 µM 2-mercaptoethanol (2-ME, Sigma, Brisbane, Australia), 5-40% calcium (NE salts, Shimizu et al., 2001), 0.5-8% iron supplement (Sigma, Brisbane, Australia), 5-40% insect supplement (Sigma, Brisbane, Australia), 0.1-10 mg/L copper II sulphate (Sigma, Brisbane, Australia), 5-40 mg/L catalase (Sigma, Brisbane, Australia), 10-20% foetal bovine serum (TropBio, Townsville, Australia), 10-20% serum supreme (Biowhittaker, Brisbane, Australia), 0-160 µg/L insulin growth factor II (IGF-II) (Invitrogen, Brisbane, Australia), 0-160 µg/L basic fibroblastic growth factor (b-FGF) (Invitrogen, Brisbane, Australia), 0-160 µg/L epidermal growth factor (EGF) (Invitrogen, Brisbane, Australia), 0-40 µg/L transforming growth factor beta (TGF-β) (Invitrogen, Brisbane, Australia), 5-40% GIT medium (Nihon Seiyaku,Tokyo, Japan), 10-80 µM dNTPs (Fermentas, Sydney, Australia), 10-20% crayfish muscle extract, 0.5-4% vitamin B (Tyca, Bordeaux, France), and 0.5-4% vitamin mix (Tyca, Bordeaux, France).

A bluegill fry cell line (BF-2) was used to represent normal cell proliferation in the WST-1 assay. BF-2 cells were cultured in Dubecco's minimum essential medium (DMEM) with 10% FBS, and incubated at 28ºC. To establish a standard proliferation curve, no cell passaging was performed during the 13-day culture.

7.3 RESULTS

7.3.1 Elemental Composition of Haemolymph

With the exception of phosphate, concentrations of all elements were lower in the culture media than in *C. quadricarinatus* and *P. monodon* haemolymph (Table 7.1). Calcium levels in the culture media ranged from 6.7-11.02 mg/dL which was lower than *C. quadricarinatus* haemolymph, 30.64 mg/dL (SD=19.8), and *P. monodon* haemolymph, 32.62 mg/dL (SD=4.6). Addition of FBS at 10% provided iron in the media at 19.39 µg/dL (SD=3.53) and copper 185 µg/dL (SD=2.57), whilst *C. quadricarinatus* haemolymph levels were 122.1 µg/dL (SD=44.8) and 235.5 µg/dL (SD=54.2) for iron and copper respectively. Similarly, *P. monodon* haemolymph values for iron and copper were also higher than the culture media, with values of 491.8 µg/dL (SD=40.9) for iron and 1865.9 µg/dL (SD=348.5) for copper.

Addition of 10× iron supplement, 10 mg/L copper, and 10% calcium (NE) salts (Appendix 2) to L-15 with 10% FBS, resulted in concentrations similar to those of *C. quadricarinatus* haemolymph.

Table 7.1. Mean concentrations (n = 10) and standard deviations (SD) for iron, calcium, copper, and phosphate in *C. quadricarinatus* and *P. monodon* haemolymph, compared to concentrations in common culture media.

	Iron (µg/dL)	**Calcium (mg/dL)**	**Copper (µg/dL)**	**Phosphate (mg/dL)**
C. quadricarinatus	122.1 (SD 44.8)	30.64 (SD 19.8)	235.5 (SD 54.2)	9.58 (SD 1.5)
P. monodon	491.8 (SD 40.9)	51.00 (SD 8.2)	1865.9 (SD 348.5)	32.62 (SD 4.6)
L-15 +10% FBS	15.86	6.7	184.5	18.74
2 x L-15 +10% FBS	19.39	8.48	188	27.77
M199 +10% FBS	22.92	11.02	183	15.63

Represents higher level in haemolymph than in culture media

Represents lower level in haemolymph than in culture media

7.3.2 Effects of Media Supplementation on Cell Proliferation

Supplementation with iron, copper, non-essential amino acids, glutaMAX™, and FBS increased proliferation of *C. quadricarinatus* cells (Table 7.2). Other supplements had a negative or no effect. Differences in cell proliferation were confirmed by visualisation. Experiments comparing effects of supplementation with growth factors (IGF-II, bFGF, EGF, TGF-β) run three times, produced conflicting and inconclusive results.

Table 7.2. Effect of culture medium supplements on proliferation of *C. quadricarinatus* cells. Shaded concentrations indicate the highest amount of replication as observed by light microscopy

SUPPLEMENT	CONCENTRATION	PEAK PROLIFERATION ABSORBANCE
Glucose	0 g/L	0.038
	1 g/L	0.034
	2 g/L	0.012
	4 g/L	0.003
	8 g/L	0.001
Lactalbumin and yeastolate	0 g/L	0.095
	0.1 g/L	0.117
	1 g/L	0.066
	2 g/L	0.058
	4 g/L	0.106
Cholesterol (250× concentrate)	0	0.046
	0.5×	0.004
	1×	0.005
	2×	0.001
	4×	0.006
Amino acids (100× concentrate)	0	0.059
	2.5×	0.058
	5×	0.07
	10×	0.099
	20×	0.099
Glutamax	0	0.02
	1 mM	0.03
	2 mM	0.03
	4 mM	0.034
	Glutamine (2 mM)	0.022
Hepes	0	0.015
	5 mM	0.009
	10 mM	0.027
	20 mM	0.002
	40 mM	0.007
Iron supplement (1000× concentrate)	0	0.005
	0.5×	0.01
	2×	0.011
	4×	0.011
	8×	0.005

SUPPLEMENT	CONCENTRATION	PEAK PROLIFERATION ABSORBANCE
Insect supplement	0	0.008
	5%	0.005
	10%	0.001
	20%	0.005
	40%	0.005
Copper	0	0.024
	0.1 mg/L	0.024
	1 mg/L	0.019
	5 mg/L	0.032
	10 mg/L	0.032
Catalase	0	0.031
	5mg/L	0.029
	10mg/L	0.025
	20mg/L	0.019
	40mg/L	0.021
Serum FBS and serum supreme	0	0.004
	10% FBS	0.012
	20% FBS	0.008
	10% SS	0.007
	20% SS	0.007
IGF-II	0	0.005
	20 µg/L	0.005
	40 µg/L	0.006
	80 µg/L	0.006
	160 µg/L	0.004
bFGF	0	0.005
	20 µg/L	0.011
	40 µg/L	0.006
	80 µg/L	0.001
	160 µg/L	0.001
EGF	0	0.007
	20 µg/L	0.004
	40 µg/L	0.003
	80 µg/L	0.005
	160 µg/L	0.009
TGF-β	0	0.004
	5 µg/L	0.004
	10 µg/L	0.005
	20 µg/L	0.007
	40 µg/L	0.006
GIT media	0	0.012
	5%	0.005
	10%	0.006
	20%	0.003
	40%	0.004

SUPPLEMENT	CONCENTRATION	PEAK PROLIFERATION ABSORBANCE
dNTPs	0	0.015
	10 µM	0.008
	20 µM	0.007
	40 µM	0.004
	80 µM	0.004
Crayfish Muscle extract	0	0.005
	10%	0.002
	20%	0.001
	10% (Heat inactivated)	0.000
	20% (Heat inactivated)	0.002
Vitamin B	0	0.022
	0.5%	0.017
	1%	0.004
	2%	0.009
	4%	0.012
Vitamin Mix	0	0.011
	0.5%	0.003
	1%	0.012
	2%	0.006
	4%	0.006
2-ME	0	0.022
	50 µM	0.005
	100 µM	0.012
	200 µM	0.007
	400 µM	0.009
NE salts (Calcium)	0	0.03
	5%	0.025
	10%	0.03
	20%	0.001
	40%	0.012

Optimised L-15 medium supplemented with iron, copper, non-essential amino acids, glutaMAX™, and FBS significantly increased cell proliferation compared to standard L-15 medium with 10% FBS (Figure 7.2, $F = 6.231$, $df = 1,19$, $p < 0.05$). The cells in the optimised L-15 medium also stayed viable longer (Figure 7.3). These results suggest (as stated by the hypothesis) that the proliferative capacity of cultured crustacean cells is not only limited by the amount of nutrients (such as iron, copper, glutaMAX and FBS), but also the absence of specific nutrients or growth promoting factors (additional amino acids).

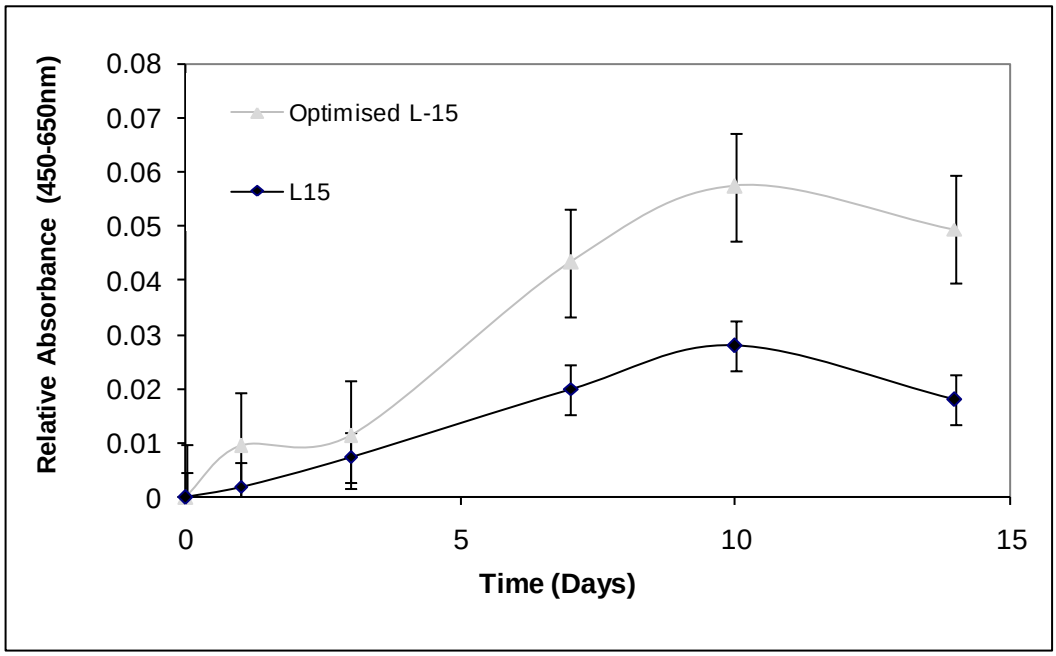


Figure 7.2. Cell proliferation in optimised L-15 culture medium compared to standard L-15 medium with 10% FBS.

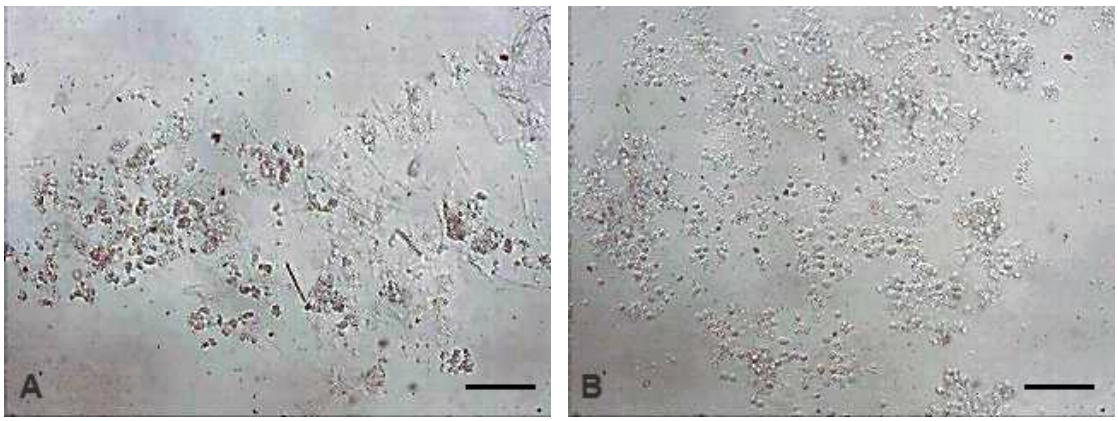


Figure 7.3. Cultured *C. quadricarinatus* cells at 25 days in standard L-15 medium with 10% FBS (A) and optimised L-15 medium (B). At 25 d, a higher number of cells were visualised in the optimised medium, which also remained viable longer. Scale bar = 80µm.

A primary cell culture from *C. quadricarinatus* exhibited a very different pattern of proliferation than the BF-2 cell line (Figure. 7.4). The latter reached a maximum cell proliferation and density within 48 h then declined as a cell monolayer was established. In contrast, *C. quadricarinatus* cell proliferation increased gradually over 13 d, and did not form a complete monolayer.

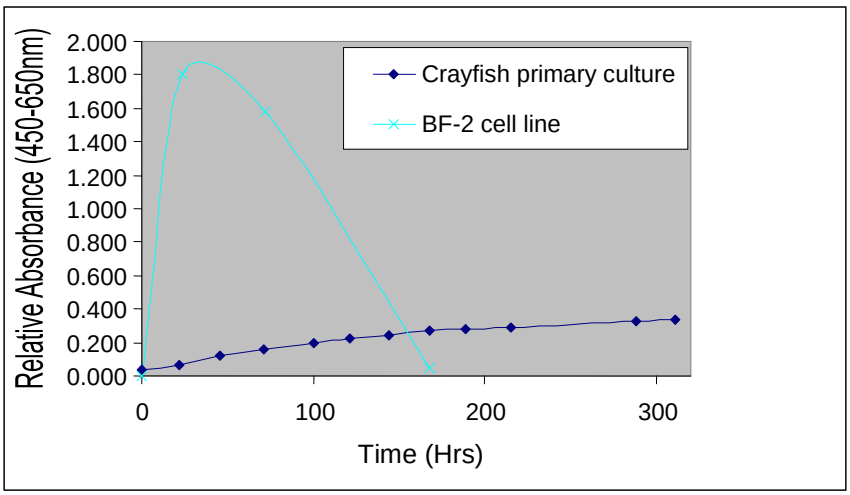


Figure 7.4. Cell proliferation in a primary cell culture for *C. quadricarinatus* and in an established cell line from bluegill fry (BF-2).

7.4 DISCUSSION

7.4.1 Elemental composition of crayfish haemolymph

Comparison of crustacean haemolymph and culture media identified nutrient deficiencies in the most commonly used cell culture media with respect to the cultivation of crustacean cells. Calcium levels, for example, were 3-8 times higher in the haemolymph of *C. quadricarinatus* and *Penaeus monodon* than in single-strength and double-strength L-15, or M199, supplemented with 10% FBS (Table 7.1). The calcium levels in crayfish and prawn haemolymph may not accurately represent concentrations in all crustaceans, as species residing in freshwater and terrestrial environments are more limited in their calcium availability than marine species (Zanotto and Wheatly, 2003). However the calcium levels of *C. quadricarinatus* haemolymph 30.64mg/dL (or 7.6mM) were within the range of calcium levels reported for other freshwater crustaceans of 6.3-16.5mM (Mothersill and Austin, 2000). It might be concluded that calcium levels should be tested and, if necessary, supplemented in media used for crustacean cell culture. Najafabadai (1992) showed that addition of sodium citrate combined with calcium chloride (NE salts) boosted the availability of calcium in L-15 medium. In the current study, 10% NE salts were added to L-15 to bring calcium up to the levels in *C. quadricarinatus* haemolymph. However,

supplementation with calcium alone did not enhance proliferation of *C. quadricarinatus* cells *in vitro* (Table 7.2).

Typical cell culture media, such as L-15 and M199, do not contain iron or copper, but these elements are provided by the addition of FBS or sera. Despite supplementation with FBS, iron levels in the three media tested were 5-30 times lower than the iron levels in *C. quadricarinatus* or *P. monodon* haemolymph. The management of iron delivery is one of the most important and complex requirements of a cell culture system (Young and Garner 1990). Iron is necessary for DNA synthesis and a host of metabolic processes (Cazzola *et al.,* 1990; Zodl *et al.,* 2005). Without iron, cells stop proliferating and eventually die, presumably because iron is required by ribonucleotide reductase and other enzymes involved in cell division (Hoffbrand *et al.,* 1976). Supplementation of L-15 with 2x and 4x iron did enhance proliferation of *C. quadricarinatus* cells. Increased availability in iron-supplemented media may allow iron complexes to form with bio-molecules, such as amino acids, providing more iron for cell functions and cell cycle progression.

Like iron, copper is an essential trace element, with copper-containing enzymes playing an important role in metabolism and free radical scavenging. It exists in the oxidation states Cu(I) or Cu^{+}(cuprous), and Cu(II) or Cu^{2+} (cupric) under physiological conditions. The shift back and forth between these two oxidation states via single-electron-transfer reactions is the property that makes copper such an essential component for enzymes (Winzerling and Law 1997). Copper levels in media with 10% FBS, were only slightly lower than in *C. quadricarinatus* haemolymph, but were 10 times lower than in *P. monodon* haemolymph. However, supplementation with 10 mg/L of copper resulted in the highest proliferation rates of *C. quadricarinatus* cells.

Limiting iron and copper *in vitro* by adding only sera to culture media may limit the number of cells able to undergo proliferation. Elemental limitation could also explain why a number of crustacean cell culture studies reported an increase in proliferation when 20% sera was added (Ellender *et al.*, 1992; Lu *et al.*, 1995a; Lu *et al.*, 1995b; Chen and Wang, 1999; Itami *et al.*, 1999; Wang *et*

al., 2000; Shimizu *et al.*, 2001; Fan and Wang, 2002; Lang *et al.*, 2002a; Lang *et al.*, 2002b; Jiang *et al.*, 2005; Luo and Dequan, 2005) or 15% sera (Kasornchandra *et al.*, 1999; Assavalapsakul *et al.*, 2003; Tirasophon *et al.*, 2005), rather than the standard 10%.

Of the four elements examined, phosphate was the only one that had higher levels in the three media tested than in haemolymph *of C. quadricarinatus.* Therefore, the culture media used to measure *C. quadricarinatus* cell proliferation was not supplemented with phosphate. However, phosphate is involved in the construction of nucleic acids (Cogan, 1999) and is considered one of the most important ions in nature (Shirahama *et al.*, 1996), so a deficiency *in vitro* could be detrimental to cellular development. Phosphate levels in *P. monodon* haemolymph were 1.2-2 times higher than those in the media. Although *P. monodon* cells were not cultured in the present study, future research could examine the effect of phosphate supplementation on prawn cells.

7.4.2 Effects of Media Supplementation on Cell Proliferation

Supplementation of L-15 with 10% FBS caused a threefold increase in proliferation of crayfish cells (Table 7.2). In addition to supplying iron and copper, vertebrate serum is a complex supplement of other minerals, lipids, and hormones, and contains growth and adhesion factors that promote cell attachment and proliferation (Freshney, 2005). The results of several studies indicated that FBS is essential for *in vitro* cell growth, although it is unlikely that FBS provides all the specific hormones and growth factors needed by crustacean cells (Mothersill and Austin, 2000; Mulford *et al.*, 2001; Crane and Williams, 2002). In vertebrate culture, growth factors are often used instead of sera (Nishino and Mitsuhashi, 1995). However, until specific growth factors for crustacean cells are identified, FBS addition will be necessary for crustacean cell culture as a source of those unidentified factors, as well as iron and copper.

Manufactured L-15 medium contains all of the essential amino acids required by crustaceans, and lacks only three non-essential amino acids: proline, glutamic acid, and aspartic acid (Claybrook, 1983). Previous research showed that

cultured prawn cells benefited from proline supplementation (Luedeman and Lightner, 1992; Toullec *et al.*, 1996; Mulford *et al.*, 2001; Shimizu *et al.*, 2001; Maeda *et al.*, 2003; Maeda *et al.*, 2004). However, other researchers added a mixture of amino acids to cultured crustacean cells with varying results (Rosenthal and Diamant, 1990; Chen and Wang, 1999). Results of the present study showed that the addition of 10% non-essential amino acids resulted in the highest cell proliferation rate of *C. quadricarinatus* cells. It is possible that the cultured cells had lost their ability to synthesise non-essential amino acids, or that synthesised amino acids were being lost through leakage into the culture medium (Freshney, 2005). Alternatively, concentrations of the non-essential amino acids in the L-15 medium may be below saturation levels. The concentration of amino acids in the medium usually limits the maximum cell concentration and thereby influences cell survival and growth rate (Freshney, 2005).

Supplementation of L-15 with Glutamax™, a substitute for L-glutamine, also enhanced proliferation of crayfish cells. L-glutamine is used by cultured cells as a source of energy and carbon (Butler and Christie, 1994), and its addition to culture media can greatly enhance cell survival (Freshney, 2005). However, L-glutamine degrades spontaneously in cell culture media, generating ammonia. Ammonia can be toxic to cells and can affect protein glycosylation, ultimately limiting cell growth. Concentrations exceeding 1 mM total ammonia are usually toxic to mammalian cells (Hrnjez *et al.*, 1999), and ammonia toxicity occurs in aquatic animals at lower concentrations (Weihrauch *et al.*, 2004). For example, the 24 h LC_{50} of NH^3-N is 0.54 mg/L (0.03 mM) for *P. monodon* nauplii (Chen and Lei, 1990). Glutamax™ is a dipeptide-stabilised form of L-glutamine that prevents degradation and ammonia build-up in cell cultures (Figure 7.5). The use of glutaMAX™ as a L-glutamine substitute in cell culture media may simply reduce ammonia production and thus, decrease toxicity and physiological stress.

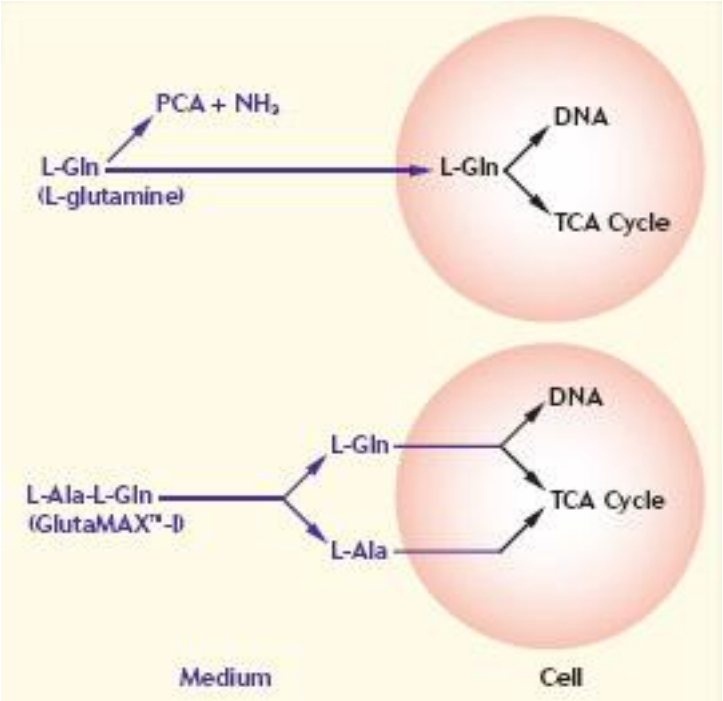


Figure 7.5. Controlled delivery of L-glutamine and its derivative, glutaMAX™, from medium to cells in culture. Source: Invitrogen, Brisbane, Australia

Previous studies reported positive effects of vertebrate growth factors on the division and behaviour of primary cultures from marine invertebrates (Mothersill and Austin, 2000). IGF-II and bFGF benefited the culture of embryonic tissue from *P. chinensis* (Fan and Wang, 2002). Basic FGF also enhanced *in vitro* survival of *P. monodon* tissues (Hsu *et al.*, 1995). EGF was successfully used as a media supplement for cultures of lymphoid organ cells from *P. vannamei* and *P. stylirostris* (Lu *et al.*, 1995a; Lu *et al.*, 1995b; Tapay *et al.*, 1995). Although visual observations of *C. quadricarinatus* cultures suggested that some of the growth factors tested were beneficial, the results of cell proliferation measurements were inconsistent, and no conclusion was reached concerning supplementation. Once a crustacean cell line is developed, it could be used to test the benefit of growth factors, which should provide more consistent results than comparing effects on separate primary cell cultures.

Aside from iron, copper, FBS, non-essential amino acids, Glutamax™, and possibly vertebrate growth factors, none of the other supplements tested enhanced proliferation of *C. quadricarinatus* cells compared to the standard L-15 medium, over the experimental period of 10 days. In contrast, results of

previous research indicated that a number of the same supplements increased cell viability and longevity in culture, including glucose (Kasornchandra *et al.*, 1999; Wang *et al.*, 2000; Mulford *et al.*, 2001; Crane and Williams, 2002; Assavalapsakul *et al.*, 2003; Gao *et al.*, 2003; Maeda *et al.*, 2003; Maeda *et al.*, 2004; Jiang *et al.*, 2005), lactalbumin and yeastolate (Maeda *et al.*, 2003; Maeda *et al.*, 2004), cholesterol (Kasornchandra *et al.*, 1999), HEPES buffer (Rosenthal and Diamant, 1990; Luedeman and Lightner, 1992; Hsu *et al.*, 1995; Toullec, 1999; Crane and Williams, 2002; Lang *et al.*, 2002a), 2-mercaptoethanol (Bannai, 1992), NE salts (Shimizu *et al.*, 2001), GIT (Jiang *et al.*, 2005), crustacean muscle extracts (Chen *et al.*, 1986; Chen *et al.*, 1988; Chen and Wang, 1999; Kasornchandra *et al.*, 1999; Owens and Smith, 1999; Mulford *et al.*, 2001), and catalase (Domart-Coulon *et al.*, 1994). However, many of these studies relied on observation of cell proliferation. It is likely, furthermore, that effective culture media supplements may be specific to the tissue, species, and culture media, and therefore, may not enhance proliferation in all crustacean cell cultures. Considering the observed differences in haemolymph composition between *P. monodon* and *C. quadricarinatus*, the optimised medium may be specific for *C. quadricarinatus* cells, and may not affect cell proliferation or longevity in other crustacean species.

7.4.3 Conclusions

The present study compared a variety of media supplements to determine their effects on proliferation of *Cherax quadricarinatus* cells in culture. The supplements included a broad range of nutrients, polypeptide growth factors, protective agents, lipids, chemical elements, and amino acids. The WST-1 assay used to measure cell proliferation in the present study provided a quick, simple, and quantitative method for comparing effects of various media supplements. However, the cell proliferation assay, as applied herein, did have limitations. First, the proliferative capacity of primary cells is much more limited than cells derived from a continuous culture. This limitation was evident when cell proliferation was compared for primary *C. quadricarinatus* cells and the BF-2 cell line (Figure 7.3). Cell lines are composed of homogeneous cells, whose rapid growth is selected for by passage. Second, the heterogeneous mixture of crayfish cells used in the present study may not all respond in the same way to

the tested supplements; a specific supplement may have a beneficial effect on some cell types, and no effect or a detrimental effect on others. A third limitation of the cell proliferation assay is that contamination can significantly affect the outcome. The initiation of primary cell cultures is susceptible to contamination; the use of antibiotics can limit, but may not eliminate, contaminant growth. Fungal and yeast contaminants are particularly difficult to control (Freshney, 2005). Therefore, aseptic primary culture techniques are imperative to ensure that positive cell proliferation is due to media supplementation and not to the growth of unidentified organisms.

The low mitotic activity of many marine invertebrate tissues constitutes the main impediment to obtaining continuous cell lines (Grace 1965; Derikot and Blinova 1982; Kawamura and Fujiwara 1995; Odintsova *et al.,* 2005). Low mitotic activity can also limit the use of cell cultures for viral isolation, as some viruses will only infect replicating cells (Kraft and Tischer 1978; Emmett *et al.,* 2004). By optimising culturing conditions, cell proliferation can be increased and *in vitro* cell longevity enhanced, allowing more opportunities for experimentation and potential manipulation and transformation.

CHAPTER 8
TRANSFECTION OF *CHERAX QUADRICARINATUS* PRIMARY CELL CULTURES

8.1 INTRODUCTION

Primary cells have limited proliferative capacity in culture, due to cellular senescence, while a cell line is a propagated culture that contains immortal cells capable of indefinite cellular division (Yamamoto *et al.*, 2003; Freshney, 2005). Although spontaneous transformation of a primary cell culture into an immortal cell line can occur, it is a rare event (Crane, 1999). This is particularly true for crustacean cell culture. Primary cell cultures from crustaceans have been initiated since the 1960s, and no permanent cell line has been made available. The lack of spontaneous transformation may be due to the lack of oncoviruses that infect these animals.

The cell cycle consists of two main phases. During interphase, the cell grows and replicates its genetic material. Interphase is separated into three stages called Gap 1 (G1), DNA synthesis (S) and Gap 2 (G2). Division of the cell into two identical cells occurs during mitosis. A cell may pause during G_1, prior to DNA synthesis, and enter a state of dormancy (G_0), which can sometimes be irreversible. Entry into the S stage is tightly regulated by signals from the environment, with cycle duration dependent upon species and cell type. In somatic cells, cellular senescence occurs after a number of progressions through the cell cycle, and the cell dies. Vertebrate somatic cells typically have a finite lifespan of approximately 20-100 generations (Freshney, 2005). This lifespan is regulated by a group of dominant genes that control products which negatively regulate cell cycle progression and limit the proliferative potential of cells (Sasaki *et al.*, 1996). Disrupting those genes should allow escape from the senescence pathway and push cells towards immortalisation and transformation (Crane, 1999).

Many viruses possess genes encoding proteins that suppress or delay apoptosis long enough for the production of viral progeny (Teodoro and Branton, 1997; O'Brien, 1998), including adenovirus, simian virus 40 (SV40),

polyomavirus, and human papillomaviruses (HPV). These viruses encode proteins that interact with key regulators of the cell cycle to stimulate unscheduled DNA synthesis. Cell cycle regulatory genes, such as p53 and the retinoblastoma (Rb) gene are the most commonly affected (Sager, 1992). The genes act at the first gap phase (G_1) checkpoint, ensuring the integrity of the genome prior to cells entering the synthesis (S) phase. p53 is usually inactive in somatic cells, and the cells proceed around the normal cell cycle (Figure 2.4). However, in response to DNA damage, such as UV radiation or oncogenes, the regulatory genes become activated by phosphorylation, often resulting in growth arrest at the G_1/S boundary or apoptosis (Sager, 1992; Levine, 1997). For continual cell proliferation, suppression of both p53 and Rb genes may be necessary.

Papillomaviruses are non-enveloped, double-stranded DNA tumour viruses that play a critical role in the formation of anogenital cancer (Putral *et al.*, 2005). Infection with high-risk HPV types (eg. HPV-16) is the predominant aetiological factor in the development of cervical cancer (Putral *et al.*, 2005). HPV's transforming capabilities arise from over-expression of the early genes, E6 and E7, which results in the integration of the viral genome into the host genome. The E6 and E7 genes are not present in normal cells, but are essential for cervical cancer development. The E6 protein binds and promotes the degradation of p53 (Scheffner *et al.*, 1990). p53 encodes a protein for tumour suppression, and has been described as "the guardian of the genome," referring to its role in preventing genomic mutation (Strachan and Read, 1999). Inactivation of p53 is the most prevalent alteration found in human and animal tumours. The HPV-16 E7 protein targets the retinoblastoma tumour suppressor protein (pRb), which it binds and destablises via ubiquitin-mediated proteosomal degradation (Boyer *et al.*, 1996). This binding action activates cell proliferation, permitting cells to pass through the G1/S boundary of the cell cycle. In cervical cancer, tumour cells rely on the continual expression of HPV E6 and E7 for growth; loss of oncogene expression results in reduced DNA replication and senescence due to the re-establishment of the tumour suppressor pathways (Goodwin *et al.*, 2000; DeFilippis *et al.*, 2003).

Previous research has shown that expression of HPV-16 E6 and E7 can efficiently immortalise different types of human somatic cells, such as fibroblasts (Shay *et al.*, 1993; Shiga *et al.*, 1997), mammary epithelial cells (Shay *et al.*, 1993; Wazer *et al.*, 1995), foreskin keratinocytes (Halbert *et al.*, 1991), smooth muscle cells (Perez-Reyes *et al.*, 1992), chondrocytes (Chen *et al.*, 2006), and oral squamous cells (Kingsley *et al.*, 2006). The neoplastic transformation of vertebrate cells was achieved by transfection with active oncogenes (Ratner *et al.*, 1985). To date, this technique has had limited application in aquatic invertebrate cells. The aims of the research described herein were to investigate a method of transfection for primary cultures of *Cherax quadricarinatus* cells and to manipulate cell cycle regulation by transfecting cells with oncogenes, with the ultimate goal of inducing an immortal cell line.

8.2 METHODS

8.2.1 Primary Cell Cultures

Two primary cell cultures were initiated from haematopoietic tissue of *C. quadricarinatus* and maintained using methods described in Section 6.2.3. These cultures included a fresh haematopoietic cell culture, which contained observable proliferating cells, and a 10 week-old culture of viable cells that had reached a state of dormancy with no observable mitosis. Both cultures were maintained in optimised L-15 medium (Section 7.3.2) and incubated in 24-well plates (TPP, Zollstrasse, Switzerland).

Cultured cells were observed daily with an Olympus IMT-2 inverted phase-contrast microscope and images captured (Section 3.6). Viable cells counts were conducting according to the procedure described in Section 3.7.

8.2.2 Transfection Vectors

8.2.2.1 Green Fluorescence Protein Vector

A green fluorescence protein (GFP) vector, pEGFP-N1, was used for initial transfection investigations (Clontech, Mountain View, USA) of primary cells (Figure 8.1). Cells exposed to pEGFP-N1 were checked for GFP expression 24 h post-transfection. Cells were sedimented by centrifugation (600 × *g* for 1 min)

in the 24-well culture plate, and the culture medium was removed to allow better fluorescence visualisation. Cells were then examined using a Leica MZFLIII fluorescence microscope.

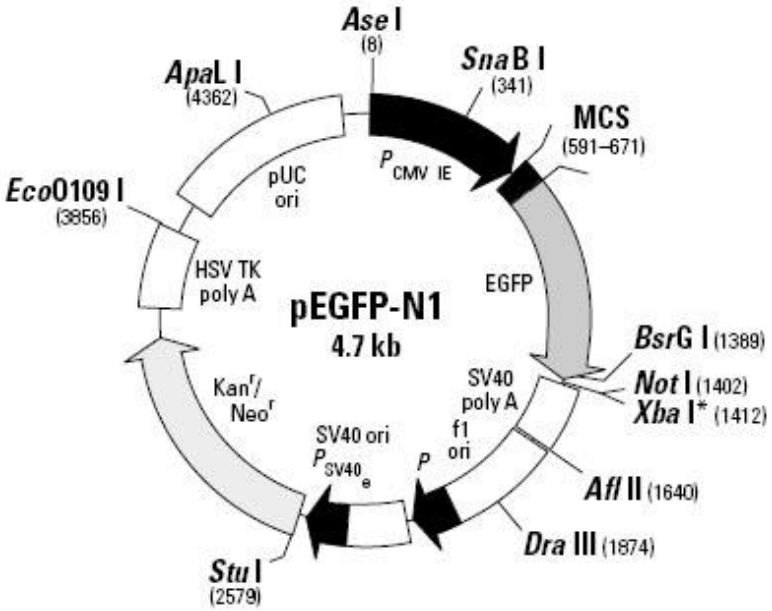


Figure 8.1. pEGFP-N1 vector map with restriction enzyme sites. The vector contains an epiflorescent GFP, along with kanamycin and neomycin resistance genes. This vector was transfected into primary cells of *C. quadricarinatus*.

8.2.2.2 Human Papilloma Virus-16 vectors

Human papilloma virus 16 (HPV-16) oncogenes E6 and E7 were supplied by Dr. Germain Fernando (CICR Princess Alexandra Hospital, Brisbane). These genes had previously been inserted into pJ4Ω vector. Two vectors were used for transfection: one contained HPV-16 E7 gene (Figure 8.2A), and the other contained the HPV-16 E6 and E7 genes (Figure 8.2B). During transport of the HPV-16 plasmids, 5 µl of each plasmid was applied to Whatman™ FTA filter paper and allowed to dry at room temperature. To retrieve the plasmid DNA, a small section of the filter paper (1-2 mm) was aseptically removed and placed in a 1.5 ml tube. The filter paper was given two quick washes (5-10 s) with 200 µl of sterile TE buffer (Appendix 2). The plasmid was eluted by incubating the filter paper for 10 min at room temperature in 5 µl TE buffer. Plasmids were cloned into HIT^{TM}competent *E. coli* cells (RBC, Taiwan). This method allowed blue/white screening to identify bacterial colonies that contained the HPV-16

plasmids (white colonies), which were selected and grown in broth culture. Target DNA was purified from the cultures, according to the protocol of Wizard plus SV minipreps (Promega, Madison, USA).

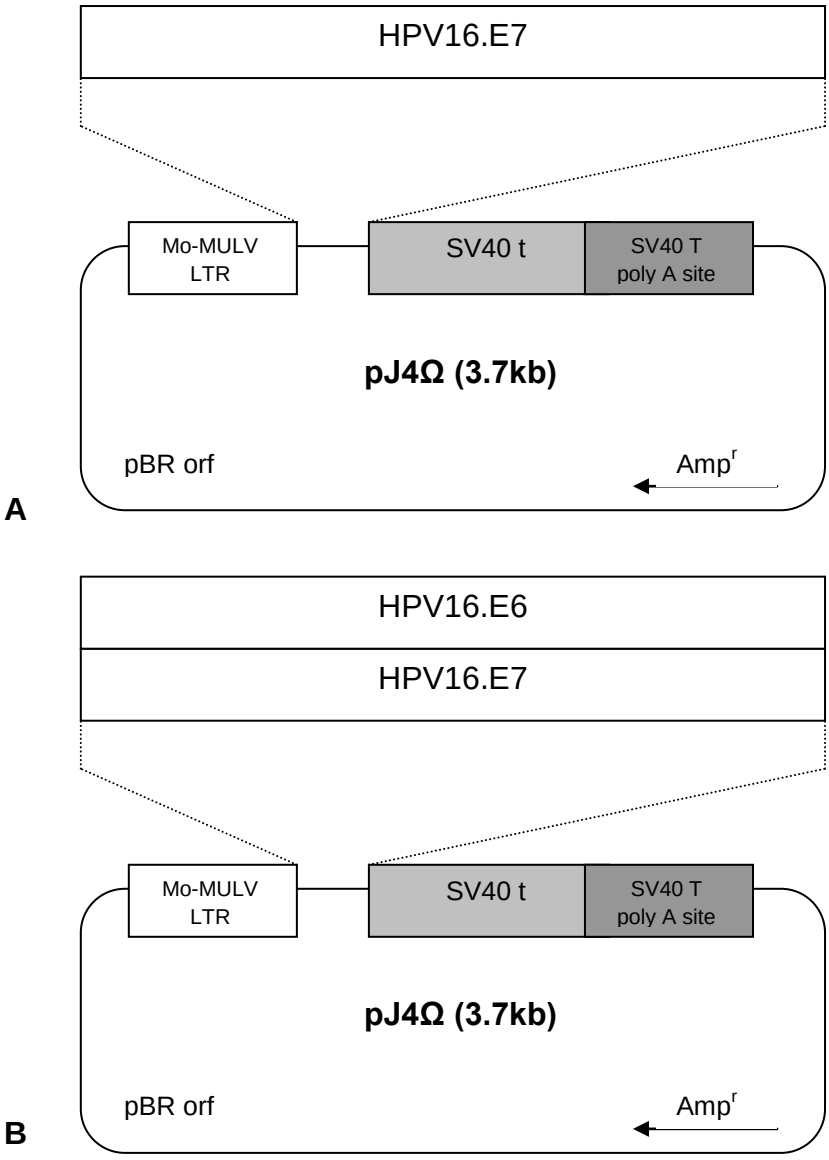


Figure 8.2. Structure of the recombinant HPV-16 DNA vectors. The expression vector, pJ4Ω, contains the Moloney murine leukaemia virus long terminal repeat (Mo-MULV LTR) as promoter, the SV40 small t intervening sequence (SV40 t) as a late promoter, and SV40 large T polyadenylation site (SV40 T poly A site). Plasmids contain either HPV-16 E7 gene (A) or HPV-16 E7 and HPV-16 E6 genes (B).

To confirm the presence of the oncogenes, PCR primers were designed using Oligo6 software, based on the E7 gene sequence (GenBank accession no. U76411, Figure 8.3). For the PCR reaction, 1 µl of the mini-prepped plasmid was added to 49 µl of PCR reaction mixture, consisting of MBI Fermentas PCR buffer (750 mM Tris-HCl, pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween20), 200 µM dNTPs, 1 µM of each primer, 1.5 mM $MgCl_2$, and 1U *Taq* polymerase (MBI Fermentas). Amplification was carried out in an Eppendorf mastercycler, with the PCR profile consisting of 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min, and a final 5 min extension at 72°C. A negative control contained no DNA template. PCR products were visualised by electrophoresis (Section 3.4.2). The expected product size was 128 bp. Following amplification, PCR products were purified and sequenced (Section 3.3).

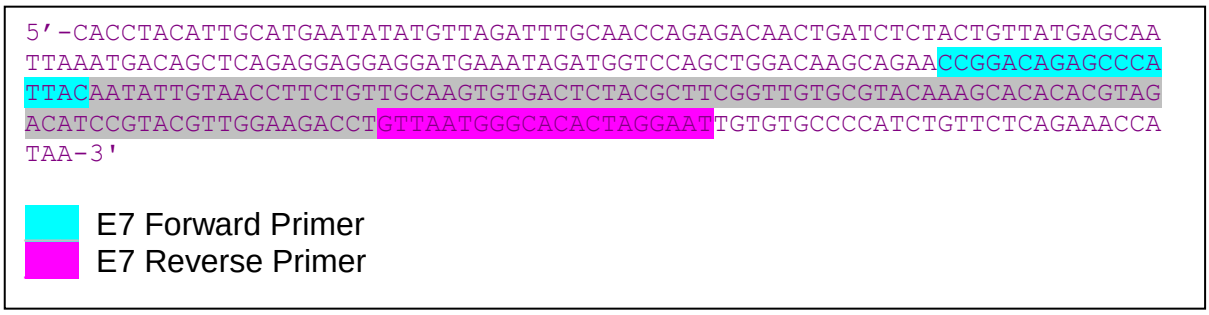


Figure 8.3. The gene sequence of HPV-16 E7. Highlighted areas represent primers designed for PCR amplification of a 128 bp product.

8.2.3 Cell Transfection

Two primary cell cultures were established from haematopoietic cells of *C. quadricarinatus*: one contained 10-week old cells, and the other contained a fresh primary culture. Cultured cells were initiated (Section 3.5) and seeded in 500 µl of optimised L-15 medium (Chapter 7) in a 24-well plate. Cells were directly inoculated with the vectors, or transfection was attempted using Lipofectamine™ LTX (Invitrogen, Brisbane, Australia). For transfection, 1.25 µl per well of Lipofectamine™ LTX were combined with 100 µl per well of transfection media (antibiotic-free L-15 medium with 2 mM glutaMAX™) and added to a 1.5 ml Eppendorf tube that contained 500 ng of oncogene vector or

GFP vector. This mixture was incubated at room temperature for 1-2 h to allow complexes to form, then added to cell cultures. For each transfection, two control groups were included: one contained primary cell cultures with no vector and no Lipofectamine™ LTX, and the other contained cells with no vector but did contain Lipofectamine™ LTX. Cells were monitored daily for morphological changes.

8.2.4 RT-PCR for mRNA

To confirm transfection with HPV-16 E6 and/or E7 genes, cellular messenger RNA (mRNA) expression was monitored at four time points: 1, 2, 10, and 14 days. At each time point, cells were removed from one well and pelleted by gentle centrifugation (500 × *g* for 5 min). Total RNA was isolated from the transfected primary cells using PureLink micro-to-midi total RNA purification system (Invitrogen, Brisbane, Australia).

Reverse transcription was performed with the SuperScript III first-strand synthesis system (Invitrogen, Brisbane, Australia), according to the manufacturer's instructions. Briefly, the RNA/primer mixture contained 200 µM of each dNTP, 0.5 µg of Oligo (dT) primer, 2 µl of RNA-extracted sample template, and sterile distilled water to give a total volume of 10 µl. Samples were incubated at 65°C for 5 min, then placed on ice for 1 min. Ten µl of reaction mixture (2 µl 10X RT buffer, 4 µl of 25 mM MgCl2, 2 µl of 0.1 M DTT), 1 µl of RNaseOUT recombinant RNase inhibitor, and 1 µl of SuperScript III RT were added to each RNA/primer mixture and incubated at 50°C for 50 min. Reactions were terminated at 85°C for 5 min before being chilled on ice. Following cDNA synthesis, the RNA template was removed from the cDNA:RNA hybrid molecules by incubation with 1 µl of RNase H for 20 min at 37°C. The cDNA was used at 10% in conventional PCR for E7 gene detection (Section 8.2.2.2). Amplified products were visualised using electrophoresis (Section 3.2).

8.3 RESULTS

8.3.1 pEGFP-N1 Transfection

Transfection of *C. quadricarinatus* primary cell cultures with pEGFP-N1 was demonstrated by the cells' ability to fluoresce. Of the two cultures transfected with the pEGFP-N1 vector, only the older, more stable haematopoietic cells demonstrated transfection. The fresh primary culture containing actively proliferating cells may not have been transfected due to the stress or "culture shock" that occurs when cells are initially explanted into culture (Lang *et al.*, 2004).

Of the 10 week-old haematopoietic cells that were successfully transfected, approximately 10-15% of cells exposed to the vector pEGFP-N1 by lipofectamine transfection took up the vector and displayed fluorescence (Figure 8.4). Thus, *C. quadricarinatus* primary cells are capable of being transfected using lipofectamine.

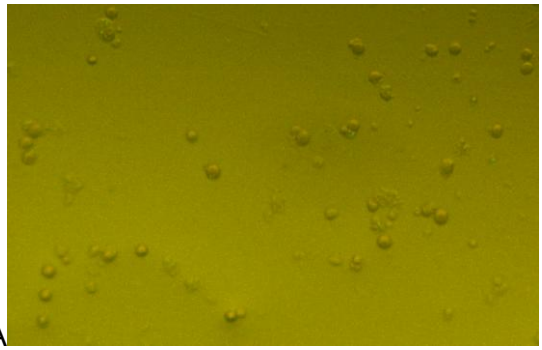
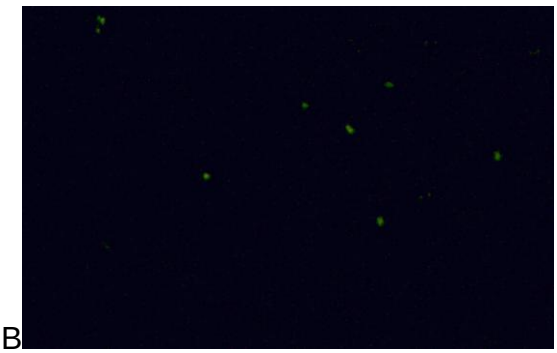
A  B 

Figure 8.4. Primary cells from hematopoietic tissue of *C. quadricarinatus* (A) visualised under light microscopy, and (B) the same view exposed to UV light displaying fluorescence. Transfection efficiency was 10–15%.

8.3.2 HPV-16 Oncogenes

The presence of the E7 gene in both pj4Ω vectors was confirmed by PCR prior to transfecting the *C. quadricarinatus* cells. Two of the four E6+E7 plasmid clones were PCR positive, and all four clones of the E7 plasmid were PCR positive (Figure 8.5). Subsequent sequencing of these amplicons (Section 3.4) matched the HPV-16 E7 DNA sequence (Genbank accession no. U76411).

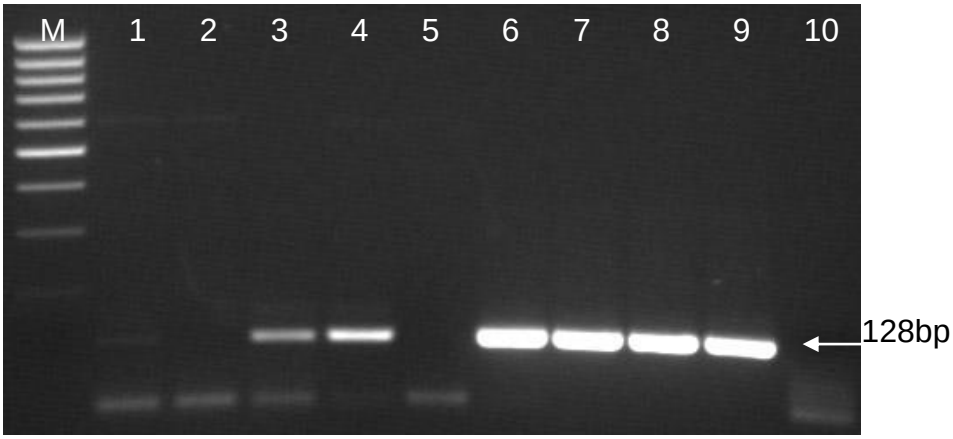


Figure 8.5. PCR of different clones of HPV-16 E6+E7 vector and HPV-16 E7 vector, using primers developed from the E7 oncogene. Lane M: GeneRuler; Lanes 1-4: HPV-16 E6+E7 clones; Lane 5: No template control; Lane 6-9: HPV-16 E7 clones; Lane 10: No template control.

8.3.3 RT-PCR for HPV-16 E7 mRNA

Results of RT-PCR verified the presence of HPV-16 E7 mRNA in the primary cells (Figure 8.6). Cells exposed to the HPV-16 E7 vector transcribed the E7 mRNA on day 2; cells exposed to the HPV16- E6+E7 vector were positive for transcribed mRNA on day 10. By day 14, all cells exposed to either HPV vector were positive for E7 mRNA. There was no difference in mRNA detection between cells exposed to the vector alone and cells exposed to the vector via lipofection.

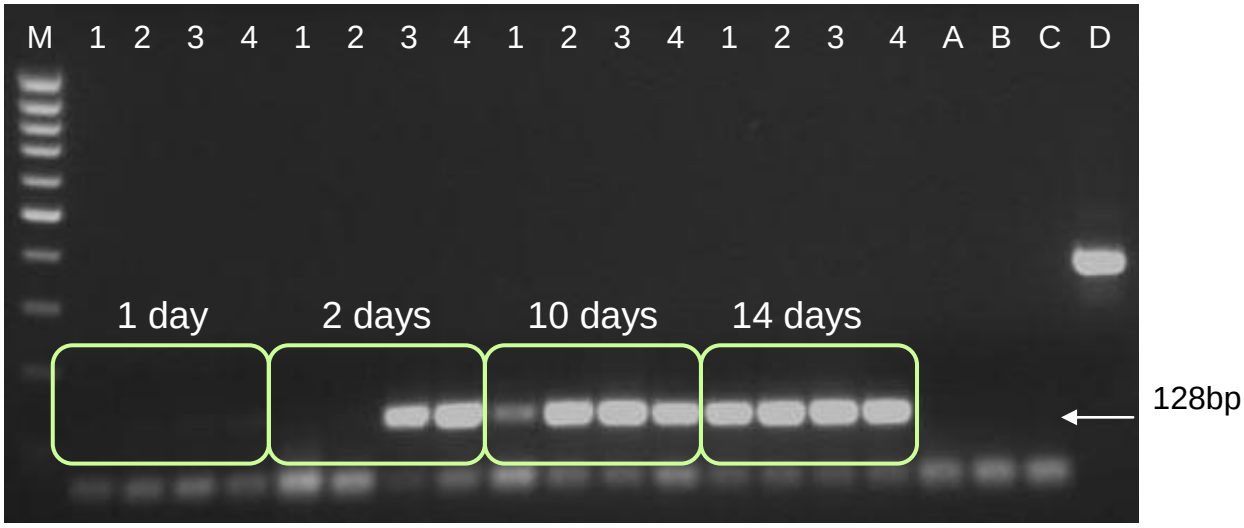


Figure 8.6. HPV-16 E7 gene detection via PCR, following cDNA synthesis of total RNA extracted from transfected cells on days 1, 2, 10 and 14 post-exposure. Lane1: Cells exposed to E6+E7 vector; Lane 2: Cells exposed to E6+E7 vector via lipofection; Lane 3: Cells exposed to E7 vector; Lane 4: Cells exposed to E7 vector via lipofection; Lane A: Cells exposed to no vectors; Lane B: Cells exposed to lipofection, but no vector; Lane C: Negative control with no template; Lane D: RT-PCR cDNA positive control (350 bp).

8.3.4 Cell Culture Morphology

Only the older, more stable haematopoietic cell cultures displayed fluorescence; therefore, only these cells were exposed to the HPV-16 oncogene vectors. Prior to transfection, the cultures consisted of semi-attached and suspended cells (Figure 8.7A). Four days after transfection, promising small cell aggregates formed (Figure 8.7B). After 150 d in culture, including 120 d of undisturbed conditions, the small aggregates remained viable (as determined by trypan blue exclusion), but no proliferation was evident (Figure 8.7C).

Figure 8.7. (A) Cultures of haematopoietic cells from *C. quadricarinatus* before transfection with HPV-16 vectors. (B) Cell aggregates began to form 4 d post-transfection with the HPV-16 E7 oncogene. (C) Viable cells remained in small aggregates (arrows) 150 d post-transfection with the HPV-16 E7 oncogene. Scale bars = 80 µm.

8.4 DISCUSSION

Much of the previous research on marine invertebrate cell cultures focused on adjustment of the culture media via supplementation to encourage continual mitosis. In mammalian cell cultures, some cell types have demonstrated spontaneous transformation *in vitro*, while others have required manipulation to assist transformation (Crane, 1999). Very few studies have investigated gene transfection and gene manipulation to assist cellular transformation. The only published research on crustacean cells transformation, was the attempted transformation of *Litopenaeus stylirostris* cells using the tumor (T) antigen gene from monkey Simian virus-40 (SV40, Tapay *et al.*, 1995), which has been used to transform mouse, rat, bovine, rabbit, hamster, and human cells (Brodsky and Pipas, 1998; MacDonald, 1990). However in the case of human cells, many of the cell lines treated with SV40-T are not truly immortal, but merely display a delay in the onset of senescence (Huschtscha and Holliday, 1983). This is largely because the SV40 large T antigen inhibits p53 by binding and stabilising it, unlike the HPV-16 E6 gene used in the present study, which both binds to and promotes degradation of p53 (Harms *et al.*, 2001). Moreover, E6 inhibits apoptosis and the transcriptional activation of p53 after DNA damage, making it a more suitable candidate for immortalisation of somatic cells.

Results of the present study demonstrated that primary cells of *C. quadricarintus* are capable of being transfected using a developed lipofection method that utilises a cationic liposome preparation, Lipofectamine™ LTX. Transfection success was initially demonstrated by the incorporation of a green fluorescent protein into the cytoplasm of 10 week-old primary *C. quadricarinatus* cells. Transfection efficiency is dependent on the type of cells as well as the method used for transfection. Typically, cell lines are much more stable and easier to transfect than primary cultures. Research has reported lipofection transfection efficiencies of 60% with human haematopoietic stem cells (Martino *et al*., 2009). For crustacean primary cells, it is unknown if 10-15% transfection efficiency is typical, as this is the first time lipofection transfection has been documented for crustacean cells. However research with *Drosophila* primary cell transfections, have reported efficiencies within this same range (Echalier, 1997). This transfection experiment demonstrated that lipofection transfection

has the potential to deliver genetic material that can take over the cells' machinery, suppress apoptosis, and encourage continual cell proliferation.

Cherax quadricarinatus primary cells transfected with HPV-16 oncogene vectors expressed E7 mRNA, suggesting that they were expressing E7 gene regulation. Transfection of the HPV-16 oncogenes occurred regardless of whether cells were transfected via lipofection or exposed to just the vector. It can therefore be concluded that the pj4Ω vector can transfect *C. quadricarinatus* haematopoietic cells without the requirement for a cationic liposome to assist transfection. Post-transfection, the cells displayed a change in morphology from a monolayer of semi-attached and suspended cells to small aggregates of cells. After 150 days in culture, however, the cells did not show an increase in proliferation. This appears to support the hypothesis that the HPV-16 E6 and/or E7 gene products by themselves are not sufficient to bring about immortalisation. However, previous studies using HPV-16 E6 and/or E7 gene products did result in transfected cells exhibiting proliferation. For example, Kingsley *et al.,* (2006) found a significant rate of proliferation in HPV-16 transfected oral squamous cells 1-3 d after exposure to the oncovirus. Yamamoto (2003) found that human embryonic fibroblast cells transfected with the HPV-16 E6 and E7 oncogenes were able to escape senescence crisis between 86 and 140 population doublings (~ 300 d) following transfection. Although no proliferation was evident, the transfected *C. quadricarinatus* cells did not show signs of senescence.

Telomere depletion may cause cells to stop dividing. Telomeres are protective DNA fragments situated at the ends of linear chromosomes, which contain repetitive DNA sequences that become shorter with each cell division, until they no longer protect the chromosome, causing the cells to cease dividing and enter a senescent state (Colgin and Reddel, 1999; Bodnar *et al.*, 1998). In many cell lines, telomere maintenance is provided by the action of the ribonucleoprotein enzyme complex, telomerase, which is expressed in germ cells and has moderate activity in stem cells, but is absent from somatic cells. Telomerase is found in most tumours. Deletions and/or mutations within senescence genes can allow cells to escape from the negative control of the cell cycle and re-

express telomerase. Telomerase activity of the *C. quadricarinatus* cells was not investigated in the current study. It may be that, although transfection of the oncogenes was successful, no proliferation was evident due to a lack of telomere maintenance. To date, only one study has been performed on telomerase activity in crustacean tissue *in vitro*. Using telomeric repeat amplification protocol (TRAP) assays, Lang *et al.,* (2004) showed active telomerase activity in cultured lymphoid organ cells of *P. japonicus* for up to 30 d. It would be of interest to measure telomerase activity in the transfected cell cultures from *C. quadricarinatus*, to determine if this is the factor limiting proliferation. Both inactivation of pRb and p53 pathways, and in combination with activation of a telomere maintenance mechanism is suggested to be necessary for immortalisation of somatic cells (Yamamoto *et al.*, 2003).

Lack of cellular attachment is another possible factor limiting proliferation of the transfected *C. quadricarinatus* cells. Kingsley *et al.,* (2006) indicated that cell proliferation of HPV-16 transfected oral squamous cells was highly correlated with an increase in adhesion. Although some small aggregates of the transfected *C. quadricarinatus* cells appeared to be semi-attached to the culture vessel, many aggregates formed in suspension. While still viable, these cells showed no signs of proliferation. When the small suspended aggregates were disrupted and sub-cultured, new small aggregates would form in suspension after 1-2 d. Kingsley *et al.,* (2006) used fibronectin to culture human oral squamous cells, and found that adhesion increased by 35-56%, which in turn increased cell proliferation. Thus, use of fibronectin could be explored as a method of enhancing cellular attachment and proliferation in cultured cells of *C. quadricarinatus* or other aquatic invertebrates.

Despite the lack of cell proliferation by *C. quadricarinatus* cells following *in vitro* transfection with oncogenes, the research reported herein demonstrated that *C. quadricarinatus* cells can successfully undergo transfection. It would be ideal for future research to combine *in vitro* oncogene transformation with measurements of telomerase activity.

CHAPTER NINE
GENERAL DISCUSSION

Over the past century, many attempts have been made to develop cell cultures from aquatic invertebrates. Of the twenty invertebrate phyla, species from six taxa have been investigated for initiation and maintenance of cells *in vitro* (Rinkevich, 2005). A large proportion of the species studied were arthropods, crustaceans in particular, largely due to their commercial importance in aquaculture and to the impact of viral diseases on this industry. Despite ongoing research, no verified crustacean cell line has been developed to date.

Previous efforts to establish a cell line capable of supporting crustacean pathogens created hybrid cells from irreversibly blocked immortal cell lines and primary cell suspensions from *Cherax quadricarinatus* and *Penaeus monodon*, with the intent of combining genetic material that would undergo continuous replication in culture and also support the growth of crustacean viruses. Interspecies cell hybrids by means of polyethylene glycol (PEG)-induced fusion for extending the life of somatic cells, has been well documented (Ogle *et al.*, 2005), with many hybrid cells being excellent tools for studying the chromosomal assignment of genes (van Olphen and Mittal, 2002). If nuclear fusion occurred in the production of the hybrid cells used in the present research, a mixture of genes from both animal cells should be present. The current study found that these hybrid cells were still viable and displayed rapid replication, but were not able to support crustacean viruses, and lacked crustacean genes and proteins. These results suggest that the hybrid cells lack some crustacean components. However, karyotyping is necessary for a more thorough investigation. Even if some crustacean genes are present, the hybrid cells will not necessarily support crustacean viral growth, as the genes and receptors from a hybrid partner can interfere with normal viral expression (Hotzel and Cheevers, 2002).

The continued failure to establish a cell line from crustacea or any other aquatic invertebrate suggests that some vital information about the physiology, biochemistry, and/or culture requirements of these cells is lacking (Rinkevich,

2005). Culture protocols developed and used for one species of aquatic invertebrates should be adaptable to other species, and even other phyla. The research described in this thesis used various tissues of the readily available freshwater crayfish, *Cherax quadricarinatus*, and a quantitative method of measuring cell proliferation, to develop an optimised cell culture medium. An increase in cell proliferation after exposure to nutrient supplements was considered an essential element in the media. Of twenty-five supplements tested, iron, copper, glutaMAX™, non-essential amino acids, and foetal bovine serum (FBS) were found to enhance proliferation of primary cell cultures.

It was also found that although the WST-1 cell proliferation assays provided a simple rapid, quantitative method of evaluating cell replication and effects of media supplementation, this method may not be the best approach for studying primary cell cultures, due to the limited proliferative capacity of primary cells compared to continuous cell lines. Furthermore, measured proliferation of primary cell cultures varied among assays, probably because a new primary culture was initiated for each assay. Finally, it was important to ensure that the primary cell cultures used in the assays were free of contaminants, as proliferation of bacterial or fungal contaminants could confound the results. In retrospect, the cell proliferation assays are more suitable for use with continuous cell cultures that contain homogenous cells and are known to be free of contaminants. Nevertheless, the optimised culture media developed using primary cultures increased not only proliferation, but also longevity and viability of *C. quadricarinatus* cells *in vitro*. Some of the initiated cell cultures, although not replicating, were still viable after twelve months. These older *in vitro* cells were then used for transfection studies, as they were less likely than fresh primary cultures to be under stress from culture initiation.

Unsuccessful attempts to establish a crustacean cell line have been attributed to lack of suitable culture medium (Mothersill and Austin, 2000), lack of specific nutrients (Najafabadi *et al.*, 1992; Shimizu *et al.*, 2001), lack of cellular attachment (Mulford *et al.*, 2001), lack of continuous mitotic division (Crane, 1999), lack of crustacean oncoviruses, and lack of a telomere maintenance mechanism (Lang *et al.*, 2004). Despite extensive efforts involving many

species, cell types, culture media, and supplements, no primary cultures of crustacean cells have transformed into permanent cell lines, strongly suggesting that artificial genetic manipulation through cell transfection is necessary. Results of the present study demonstrated that primary cells of *C. quadricarinatus* are capable of being transfected using a lipofection method that utilises a cationic liposome preparation Lipofectamine™ LTX. Successful transfection was demonstrated by the incorporation of a green fluorescent protein into the cytoplasm of 10 week-old primary *C. quadricarinatus* cells. Therefore, this technique was proven useful for delivering genetic material, and has the potential to take over the cells' machinery, suppress apoptosis, and encourage continual cell proliferation.

It has been suggested that the lack of continuous proliferation of aquatic invertebrate cells is due to senescence (Crane, 1999; Mothersill and Austin, 2000), which may result from inactivation of proliferation-promoting genes and/or activation of anti-proliferation genes, a decrease in the level of DNA methylation, telomere shortening, and/or DNA damage and repair mechanisms (Crane, 1999). Inactivation of anti-proliferation genes can occur through genetic alterations or through interaction of gene products with cellular or viral proteins (Lee and Cho, 2002). Inactivation of anti-proliferation genes in primary cells of *C. quadricarinatus* was attempted by transfecting HPV-16 oncogenes, E6 and E7. These genes interact with key regulators of the cell cycle, p53 and the retinoblastoma (Rb), to stimulate unscheduled DNA synthesis (Putral *et al.*, 2005). Inactivation of p53 is the most prevalent alteration found in human and animal tumours (Ko and Prives, 1996; Levine, 1997). Following transfection of the HPV-16 E6+E7 genes or the HPV-16 E7 gene into the *C. quadricarinatus* cells, mRNA of the E7 gene was monitored via RT-PCR. Results showed that the cells were successfully transcribing the E7 gene, but still were not undergoing continuous cell proliferation. Thus, although the transfected cells may have overcome the anti-proliferation genes that limit cell cycle progression, they still appeared to lack a mechanism required for continuous replication, such as telomere maintenance.

In somatic cells, telomere length decreases with each cell division. It is believed that when some telomeres become sufficiently short, the cells cease dividing and enter a senescent state (Bodnar *et al.*, 1998). Telomere length can be maintained in transformed or carcinoma cells by the enzyme, telomerase. Telomerase is a reverse transcriptase composed of two primary components, the proteincatalytic subunit, TERT, and the template RNA subunit, TR (McChesney *et al.*, 2000). Introduction and over-expression of the human catalytic subunit of telomerase (hTERT) in normal fibroblasts and epithelial cells leads to telomerase activity, elongation of telomeres, and extension of cellular life span (Meyerson *et al.*, 1997; Bodnar *et al.*, 1998). Telomere length in cancer cells is thought to be regulated by this telomerase complex and the proteins that bind to the telomeric DNA repeats (McChesney *et al.*, 2000). Some researchers believe that manipulation of telomerase in somatic cell culture is necessary for the development of cell lines (Bodnar *et al.*, 1998). Therefore, future research could introduce excessive telomerase to cultured crustacean cells, to potentially re-establish depleted telomere units on the chromosomes. Telomerase activity can then be monitored in cells using telomeric repeat amplification protocol (TRAP) assays (Lang *et al.*, 2004).

The research described in this thesis attempted to develop a continuous cell line from crustacean cells, using novel technologies. Although a permanent crustacean cell line was not ultimately developed, new knowledge and methodological advances were made with respect to hybridisation of crustacean cells, optimisation of culture media using metabolic assays, and transfection of crustacean cells in primary cultures. Perhaps most importantly, crustacean cells successfully transfected with HPV oncogenes are still viable at the time of this report, 270 days post-transfection. Many insect cell lines have remained dormant for long periods before exhibiting proliferation (Lynn, 1999). There is therefore a chance that the transfected crustacean cell lines established during the present study may overcome senescence and transform into continuous cell lines.

The development of a permanent *in vitro* model for crustacean species is imperative (Spann and Lester, 1997). Crustacean aquaculture is essential to

meet future global demands for seafood, particularly because most capture fisheries are at or near their production limits (FAO, 2006). However, the problems posed by viral diseases continue to plague this industry. In addition to known viral pathogens, newly discovered and emerging viruses will pose future threats to crustacean aquaculture. Cultured cell lines are now considered indispensable tools for studying the molecular and cellular mechanisms of pathogenesis in other animals (Bhunia, 2007). Cell cultures not only aid in diagnosis and identification of pathogens, but also allow analysis of interactions between viruses and host cells, and identification of the mechanisms involved in viral infection (Villena, 2003). Understanding these mechanisms may someday allow us to prevent viral infections in aquaculture populations.

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APPENDIX 1

Animal Ethics Approval

JAMES COOK UNIVERSITY
Townsville Qld 4811 Australia

Tina Langford, Ethics Administrator, Research Office. Ph: 07 4781 4342; Fax: 07 4781 5521

ETHICS REVIEW COMMITTEE
(Animal Ethics Sub-Committee)
APPROVAL FOR ANIMAL BASED RESEARCH OR TEACHING

PRINCIPAL INVESTIGATOR	**Kerry Claydon**
SUPERVISORS	Associate Professor Leigh Owens, Dr Graham Burgess
SCHOOL	Biomedical Sciences
PROJECT TITLE	**Crustacean cell culture**

APPROVAL DATE	18 May 2004	EXPIRY DATE	31 Dec 2007	CATEGORY	2

This project has been allocated Ethics Approval Number with the following conditions: **A 908**

1. All subsequent records and correspondence relating to this project must refer to this number.
2. That there is NO departure from the approved protocols unless prior approval has been sought from the Animal Ethics Sub-Committee.
3. The Principal Investigator is to advise the responsible Ethics Monitor appointed by the Ethics Review Committee:
   - periodically of the progress of the project;
   - when the project is completed, suspended or prematurely terminated for any reason.
4. In compliance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, and the *Queensland Animal Care and Protection Act 2001*, it is **MANDATORY** that you provide an annual report on the progress of your project. This report must also detail animal usage, and any unexpected event or serious adverse effect that may have occurred during the study.

NAME OF RESPONSIBLE MONITOR	Summers, Prof Phillip
EMAIL ADDRESS	phillip.summers@jcu.edu.au
ASSESSED AT MEETING APPROVED	**Date:** 4 May 2004 **Date:** 18 May 2004
[forwarded by email without signature] Tina Langford Ethics Administrator **Research Office** Tina.Langford@jcu.edu.au	Date: 18 May 2004

APPENDIX 2

Buffers and Solutions

TN Buffer

Tris-HCl (20mM)	1.2g
NaCl (400mM)	11.69g

Make up fresh with deionised water to 500mL, adjust pH to 7.4, autoclave and store at room temperature.

TE Buffer

Tris-HCl (10mM)	0.606g
EDTA (0.1mM)	0.019g

Make up with deionised water to 500ml, adjust pH to 8.0. Sterilise and store at room temperature.

Phosphate buffered saline with Tween 20 (PBS-T)

KCl	0.2g
NaCl	8g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
Tween	1%

Make up with deionised water to 800mL, adjust pH to 7.4. Adjust volume to 1L, sterilise and store at 4°C.

Modified Alsever's Solution (Anti-coagulant)

$Na_3C_6H_5O_7$ (27mM)	0.79g
NaCl (336mM)	1.96g
Glucose (226mM)	4.07g
EDTA (9mM)	0.34g

Make up with deionised water to 100ml, adjust pH to 7. Autoclave and store at room temperature.

NE Salt Solution (to increase calcium)

$C_6H_5Na_3O_7$	0.512g
$CaCl_2$	0.147g
$MgCl_2$	0.135g

Make up with deionised water to 10mL. Sterilise NE salt solution but filtering through a 0.22µ filter.

APPENDIX 3
Key Crustacean Cell Culture Studies

Species	Tissue	Culture Media	Supplementation	Reference
P. chinensis	Haemocytes	2 × L-15(*) 3 × L-15 4 × L-15	20% GIT medium (*) 2% Glucose (*) 20% FBS (*) AKN salt solution Medium for penaeid shrimp	(Jiang *et al.*, 2005)
P. monodon	LO	2 × L-15(*)	15% FBS (*) 15% Shrimp extract 5g/L NaCl (*) 5% lactalbumin (*)	(Tirasophon *et al.*, 2005)
P. japonicus	Ovary(*)	2 × L-15(*)	10% FBS (*) Glucose(*) Proline(*) Yeastolate(*) Lactalbumin hydrolysate (*)	(Maeda *et al.*, 2004)
H. americanus	Neuronal tissues	L-15	Lobster saline Vitamins	(Stepanyan *et al.*, 2004)
P. monodon	LO(*)	2 × L-15(*)	15% FBS (*) 1% Glucose (*) 5g/L NaCl (*) 5% lactalbumin (*)	(Assavalapsakul *et al.*, 2003)
P. japonicus	Ovary(*)	Eagle's MEM M199 L-15 2 × L-15 (*) Grace's Mitsuhashi & Maramorosch Insect pathology laboratory-41 TC-100	Glucose (*) Trehalose, fructose, maltose Sucrose Proline (*) Taurine Lactalbumin hydrolysate (*) Yeastolate (*) Ascorbic acid 10% FBS (*)	(Maeda *et al.*, 2003)

Species	Tissue	Culture Media	Supplementation	Reference
P. monodon *P. japonicus*	Ovary (*) Testes Heart Hepatopancreas Hepatopancreas membrane Muscle Epidermis Eye and Eyestalk Antenna Gut LO (*) Pleopods Nauplii Embryonated eggs (*)	L15 (various modifications) 2 x L15 (*) M199 Eagle's MEM L. West media Seawater modified medium	Pipes buffer Chen salts NaCl (*) Glucose (*) 10mM Hepes (*) Glutamine (*) Antibiotics 10% FBS (*) bFGF	(Crane and Williams, 2002)
P. chinensis	Embryonic tissues	Medium for Penaeid shrimp	20% FBS (*) NaCl (*) IGF-II (*), bFGF (*) Chitosan (*)	(Fan and Wang, 2002)
P. monodon	Hepatopancreas (*) Eyestalk Eye Muscle Haemolymph	L-15	Antibiotics 10% FBS	(Uma *et al.*, 2002)
P. japonicus	LO (*) Ovary (*) Heart Hepatopancreas Epithelium Muscle Ganglion	M199 (*)	Saline components (*) 10mM HEPES (*) Glutamine (*) Lactalbumin hydrolysate (*) 20% FBS (*)	(Lang *et al.*, 2002b)
P. japonicus	LO	M199	Saline components (*) 10mM HEPES (*) Glutamine (*) Lactalbumin hydrolysate (*) 20% FBS (*)	(Lang *et al.*, 2002a)

Species	Tissue	Culture Media	Supplementation	Reference
P. monodon *P. merguiensis*	LO (*) Haematopoietic cells (*) Haemocytes (*) Ovary and Testes Heart Hepatopancreas Muscle Nerve tissue	L-15 (*) Commonwealth Scientific Laboratories DMEM RPMI Bio-Rich1 ExCell 401 TC-100	Antibiotics 10% FBS (*) Chen salts Itami salts (*) Synthetic salt	(Roper *et al.*, 2001)
Nehrops norvegicus	Haematopoietic tissue	2 x L-15 (in Seawater) with Glutamine	10% FBS (*) 5% Nephros serum (*) 5% Nephros muscle extract (*) Proline (*) Taurine Thioctic acid Ascorbic acid EGF, bFGF, IGF-I 20-hydroxyecdysone Chen salts Glucose (*) Various serums Various attachment factors	(Mulford *et al.*, 2001)
P. stylirostris	Ovary cells	2 × L-15 (*) M199	Hanks salts Taurine Proline L-lysine L-glutamine NaCl, KCl, $CaCl_2$, $MgCl_2$ $ZnCl_2$, $SrCl_2$, NaBr 20 % FBS	(Shimizu *et al.*, 2001)
Orconectes limosus	Neuronal tissue (*) Gills Muscle Eggs	Eagle's MEM	6% FBS Glutamine Earle Salts	(Neumann *et al.*, 2000)
P. monodon	LO(*)	2 × L-15(*) NCTC 135 Modified Grace's	Glucose (*) Salts 20% FBS(*)	(Wang *et al.*, 2000)

Species	Tissue	Culture Media	Supplementation	Reference
P. monodon *P. penicillatus* *P. japonicus*	LO (*) Ovary Heart Hepatopancreas Haemocytes	2 × L-15 (*) M199 CMRL-1066 (*) MEM	20% FCS (*) NE Amino acids E Amino acids Lobster haemolymph Prawn ovary & muscle extract (*) Artemia extracts	(Chen and Wang, 1999)
P. monodon *P. merguiensis* *P. ensis*	Epidermis (*) Heart Ovary LO Hepatopancreas Nerve	L-15 2 × L-15 (*) M199 Iscove's MEM	10% FBS (*) Prawn muscle extract (*) Haemolymph extracts Human serum Cholesterol Galactose Trehalose Cell-Tak	(Owens and Smith, 1999)
P. monodon	Ovary (*)	2 × L-15 (*) Grace's medium	5-20% FBS (*) EGF bFGF Vitamins stock Mineral stock Prawn muscle extract Prawn haemolymph Tryptose phosphate broth Chen salts Hanks salts (*)	(Fraser and Hall, 1999)
P. monodon	LO (*) Ovary Heart	2 × L-15 (*)	15% FBS (*) 1% Glucose (*) 5g/L NaCl (*) 15% Shrimp meat extract (*) 0.01% Cholesterol (*)	(Kasornchandra *et al.*, 1999)
P. monodon	Haematopoietic tissue LO Ovary	Customised medium	Not specified	(West *et al.*, 1999)
Liocarcinus depurator *Carcinus maenas*	Haemocytes	L-15 (*) RPMI MEM	0.4M NaCl (*) 10% FCS (*) Antibiotics (*)	(Walton and Smith, 1999)

Species	Tissue	Culture Media	Supplementation	Reference
P. japonicus	LO	M199	20% FBS Lactalbumin Glutamine Crab muscle extract Crab haemolymph extract Additional salts	(Itami *et al.*, 1999)
P. monodon *P. indicus* *Emerita asiatica*	Antennae Eye Eyestalk Gills Muscle	L-15 Eagle's MEM	Ciproflaxacin	(Purushothaman *et al.*, 1998)
P. japonicus *P. stylirostris*	LO	Unspecified	Not specified	(Tapay *et al.*, 1997)
Macrobrachium rosenbergii	Embryos	Eagle's MEM L-15 2 × L-15 McCoy's 5A medium M199 RPMI-1640 Grace's medium Insect pathology laboratory-41 TC-100 Mitsuhashi & Maramorosch	5-20% FBS (*) 5M NaCl (*)	(Frerichs, 1996)
P. vannamei *P. indicus*	Epidermis Regeneration buds Hepatopancreas Ovaries Embryos	L-15 M199 Grace's medium	10% FBS 0.06g/L L-proline 10 mM HEPES Glutamine Various salt solutions	(Toullec *et al.*, 1996)
P. chinensis	Embryos Larvae Eye Heart (*) LO (*) Ovary Cerebral ganglia	2 × L-15 Grace's medium Medium for Penaeid shrimp (*)	FBS (*) Prawn muscle extract Haemolymph	(Tong and Miao, 1996)

Species	Tissue	Culture Media	Supplementation	Reference
P. monodon	LO (*) Gills Ovary Hepatopancreas Heart Muscle	L-15 (*) M199 DMEM	10% FBS (*) 5g/L NaCl (*) Glucose Sucrose EGF, TGF, IGF-I, bFGF (*) 20mM HEPES Trehalose Proteinase K Arginine Methionine Ascorbic acid	(Hsu *et al.*, 1995)
P. vannamei	LO	2 × L-15	20% FBS 4% Shrimp extract 30ng/ml EGF	(Lu *et al.*, 1995b)
P. vannamei *P. stylirostris*	LO	2 × L-15	20% FBS 4% shrimp extract 30ng/ml EGF	(Lu *et al.*, 1995a)
P. stylirostris	LO	2 × L-15	20% FBS 8% shrimp extract 20ng/ml EGF	(Tapay *et al.*, 1995)
P. vannamei *P. stylirostris*	Muscle Heart Gil Gut Nerve LO Ovary	2 × L-15 M199 2 x M199 RPMI – 1640 Grace's medium TC-100 Mitsuhashi & Maramorosch	10-20% FBS 8% shrimp extract 20ng/ml NGF or EGF	(Nadala *et al.*, 1993)
P. vannamei *P. aztecus*	Haemocytes	2 × L-15 F12 DMEM Eagle basal medium Grace's medium	20% FBS Salt solutions Glutamine Asparagine Taurine Carnitine Betaine Trimethyl amine oxide Chlorine hydrochloride	(Ellender *et al.*, 1992)

Species	Tissue	Culture Media	Supplementation	Reference
P. vannamei *P. stylirostris*	Ovary (*)	M199 L-15 Amphibian culture Grace's medium (*) Schneider's medium	10% FBS (*) 2mg/ml proline (*) 2M MgCl (*) 5M NaCl (*) Hank salts (*) 20mM HEPES (*)	(Luedeman and Lightner, 1992)
P. aztecus	Haemocytes Hepatopancreas LO (*)	2 × L-15 (*)	Glutamine Lobster haemolymph L- asparagine Taurine Carnitine Betaine Trimethylamine-N-oxide Choline hydrochloride Sodium pyruvate FBS Chen salts Salt supplement (NE)	(Najafabadi *et al.*, 1992)
P. monodon	Hepatopancreas Heart Haemolymph	2 × L-15	Prawn muscle extract	(Fuerst *et al.*, 1991)
P. semisulcatus	Haematopoietic tissue LO Hepatopancreas Ovary	Eagle's MEM Ham F-12 L-15 M199 (*)	5-20% FBS 5-20% shrimp haemolymph Glutamine 20mM HEPES Vitamin solution Amino acid mix	(Rosenthal and Diamant, 1990)
P. orientalis	Hepatopancreas	M199	15% FBS	(Hu, 1990)
P. japonicus	LO	RPMI-1640 L-15 2 × L-15 Eagle's MEM M199 CMRL-1066	18% calf serum Shrimp muscle extract Haemolymph Modified Chen's salts	(Itami *et al.*, 1989)

Species	Tissue	Culture Media	Supplementation	Reference
H. americanus	Gonad Haematopoietic	M199	10% FBS 20-Hydroxyecdysone	(Brody and Chang, 1989)
P. monodon	LO	2 × L-15	20% FCS Chen salts	(Chen and Kou, 1989)
P. monodon	Haematopoietic Ovary	2 × L-15	5-20% FBS Shrimp muscle extract (30%)	(Chen *et al.*, 1988)
P. monodon	Gill Nerve Gonad Muscle Heart Hepatopancreas	Eagle's MEM L-15 2 × L-15 NCTC 135 Various insect media Amphibian medium	10-20% FBS Haemolymph (10%) Horse serum Shrimp muscle extract 6g/L NaCl	(Chen *et al.*, 1986)

* Indicates most successful tissue, media and/or supplement (if indicated in research)

APPENDIX 4

Statistical Results for Optimised Culture Media

UNIVARIATE ANALYSIS OF VARIANCE

Between-subjects factors

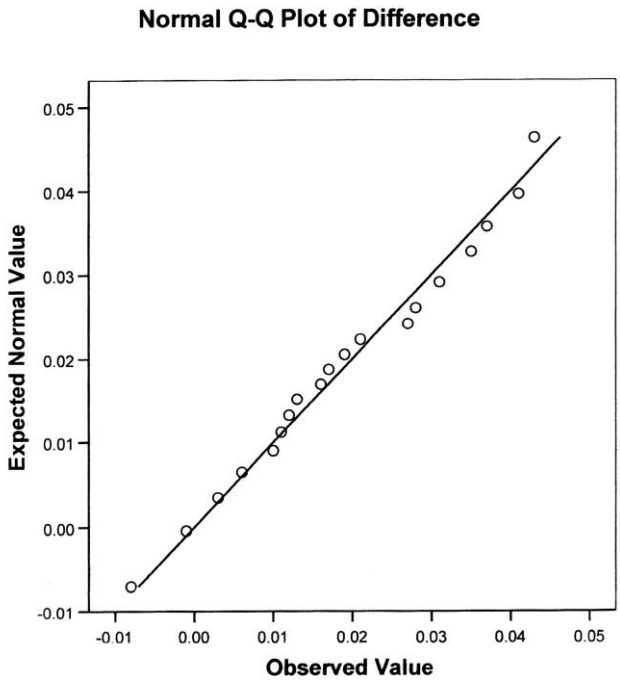
		Value Label	N
Media	1.00	L15	10
	2.00	Specialised Media	10
Day	1.00		4
	3.00		4
	7.00		4
	10.00		4
	14.00		4

Tests of Between-Subjects Effects

Dependent Variable: Difference

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.003[a]	9	.000	5.499	.007
Intercept	.008	1	.008	118.203	.000
Media	.000	1	.000	6.231	.032
Day	.003	4	.001	10.247	.001
Media * Day	.000	4	3.69E-005	.567	.692
Error	.001	10	6.50E-005		
Total	.012	20			
Corrected Total	.004	19			

a. R Squared = .832 (adjusted R Squared = .681)



POST HOC TESTS

Multiple Comparisons

Dependent Variable: Difference
LSD

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	3.00	-.0058	.00570	.337	-.0185	.0070
	7.00	-.0223"	.00570	.003	-.0350	-.0095
	10.00	-.0308"`	.00570	.000	-.0435	-.0180
	14.00	-.0230*	.00570	.002	-.0357	-.0103
3.00	1.00	.0058	.00570	.337	-.0070	.0185
	7.00	-.0165"	.00570	.016	-.0292	-.0038
	10.00	-.0250*	.00570	.001	-.0377	-.0123
	14.00	-.0173'`	.00570	.013	-.0300	-.0045
7.00	1.00	.0223*	.00570	.003	.0095	.0350
	3.00	.0165*	.00570	.016	.0038	.0292
	10.00	-.0085	.00570	.167	-.0212	.0042
	14.00	-.0008	.00570	.898	-.0135	.0120
10.00	1.00	.0308*	.00570	.000	.0180	.0435
	3.00	.0250*	.00570	.001	.0123	.0377
	7.00	.0085	.00570	.167	-.0042	.0212
	14.00	.0078	.00570	.204	-.0050	.0205
14.00	1.00	.0230*	.00570	.002	.0103	.0357
	3.00	.0173*	.00570	.013	.0045	.0300
	7.00	.0008	.00570	.898	-.0120	.0135
	10.00	-.0078	.00570	.204	-.0205	.0050

Based on observed means.
*. The mean difference is significant at the .05 level.

APPENDIX 5

Presentations and Publications

Poster Presentation

- Claydon, K. and Owens, L. (2006). Mitosis in a bottle. Townsville Research Festival, Townsville, Australia

Oral Presentation

- Claydon, K. and Owens, L. (2008). Immortalisation of crustacean cells using human cancer genes? Diseases of Asian Aquaculture VII, Taipei, Taiwan.

Journal Publications

- Claydon, K. and Owens, L. (2008) Attempts at immortalization of crustacean primary cell cultures using human cancer genes. In Vitro Cellular Development Biology – Animal 44:451-457

- Claydon, K. and Owens, L (2009). Optimisation of *in vitro* cell proliferation using colourmetric assays for *Cherax quadricarinatus* culture media. Cytotechnology (Submitted)

- Claydon, K., Roper, K. and Owens, L. (2009). Attempts at producing a hybridised *Penaeus monodon* cell line by cellular fusion. Fish and Shellfish Immunology (Submitted)