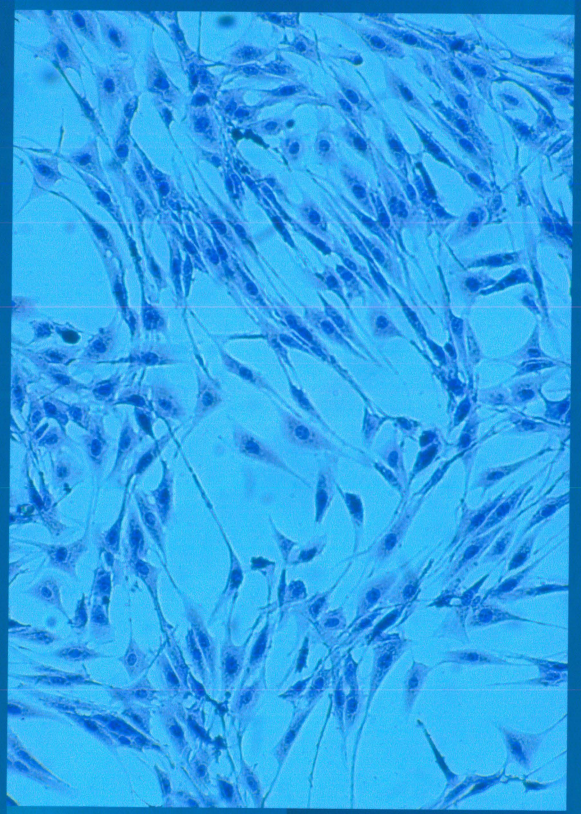
Development of Continuous Prawn Cell Lines for Virus Isolation and Cultivation

Mark S. Crane and Lynette M. Williams



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Project No. 1997/222

Development of continuous prawn cell lines for virus isolation and cultivation

Mark S. Crane and Lynette M. Williams

Published by CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong

ISBN 0 643 06833 3

Formatted/designed by Frank Filippi, CSIRO Livestock Industries
Printed by CSIRO Livestock Industries

TABLE OF CONTENTS

Project Investigators			ii
Acronyms			iii
List of Figures and Tables			iv
1.0	NON-TECHNICAL SUMMARY		1
2.0	ACKNOWLEDGEMENTS		3
3.0	INTRODUCTION		4
	3.1	Background	4
	3.2	Need	5
	3.3	Objectives	6
4.0	METHODS		7
	4.1	Juvenile and Adult Prawns	7
	4.2	Eggs and Nauplii	7
	4.3	Tissue Dissociation	8
	4.4	Primary Cell Cultures	10
	4.5	Insect Cell Cultures	14
	4.6	White Spot Virus	15
	4.7	Cell Proliferation Assay	15
5.0	DETAILED RESULTS		17
	5.1	Primary Cell Cultures derived from Prawn Tissues	17
	5.2	Disinfection of Nauplii and Eggs	21
	5.3	Nauplii Cell Cultures	21
	5.4	Primary Cell Cultures derived from Embryonated Eggs	21
	5.5	Insect Cell Cultures	25
	5.6	Cell Proliferation Assay	27
6.0	DISCUSSION		29
	6.1	Introduction	29
	6.2	Prawn Tissues	30
	6.3	Cell Culture Medium	30
	6.4	Cell Transformation	30
	6.5	Conclusions	31
7.0	BENEFITS		36
8.0	INTELLECTUAL PROPERTY		36
9.0	FURTHER DEVELOPMENT		36
10.0	STAFF		37
11.0	DISTRIBUTION		38
12.0	BIBLIOGRAPHY		40

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PROJECT TITLE

Development of continuous prawn cell lines for virus isolation and cultivation

PROJECT NUMBER

97/222

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ACRONYMS

Acronym	Full Name
AAHL	Australian Animal Health Laboratory
AIMS	Australian Institute of Marine Science
ATA	3-amino-1, 2, 4-triazole
bFGF	basic fibroblast growth factor
BME	basal medium Eagle
BrdU	5-bromo-2'-deoxy-uridine
Cat #	catalogue number
CLI	CSIRO Livestock Industries
CMR	CSIRO Marine Research
CRC	Collaborative Research Centre
CSIRO	Commonwealth Scientific & Industrial Research Organisation
DNA	deoxyribonucleic acid
EMEM	Eagle's minimun essential medium with Earle's salts
FBS	foetal bovine serum
g	gram
G	gauge
HAT	Hypoxanthine Aminopterin Thymidine
HGPRT	hypoxanthine guanine phosphoribosyl transferase
IU	international units
L-15	Leibovitz L-15 medium
M-199	medium 199
mL	millilitres
mM	millimoles
ng	nanogram
µg	micrograms
µL	microlitres
µM	micromoles
OIE	Office International des Epizootie
PBS	phosphate buffered saline pH 7.4
PCR	polymerase chain reaction
p.i.	post-infection
pL	post-larvae
ppm	parts per million
QDPI	Queensland Department of Primary Industry
SPF	specific pathogen free
v/v	volume per volume
WSS(V)	white spot syndrome (virus)
w/v	weight per volume

LIST OF FIGURES AND TABLES

FIGURES

Figure 1. 4-day-old prawn lymphoid organ cells in 96-well plate (100X magnification).

Figure 2. Photomicrograph of 4-day-old prawn lymphoid organ cells in 24-well plate (100X magnification).

Figure 3. Photomicrograph of 6-day-old prawn lymphoid organ cells in 96-well plate (100X magnification).

Figure 4. Vitellin cells from embryonated cells treated with 0.5mM ATA and put through a mesh.

Figure 5. Reaction curves for the real-time PCRs for each of the samples analysed

Figure 6. Photomicrograph of pilchard liver cell cultures labelled with BrdU (400X magnification).

Figure 7. Photomicrograph of cell cultures of the rainbow trout gonad cell line RTG-2 labelled with BrdU (400X magnification).

TABLES

Table 1. Formulations of cell culture medium used in initial experiments

Table 2. Modified cell culture media used for primary cell cultures

Table 3. Summary of pH and Osmolarity Measurements of various formulations of Leibovitz's L-15 Medium

Table 4. Summary of ATA treatments used to remove fertilisation membrane

Table 5. Summary of real-time PCR analysis of cell culture supernatants from WSSV-inoculated Sp-21 cell cultures

Table 6. Summary of recent studies on prawn cell culture

1.0 NON-TECHNICAL SUMMARY

97/222 **Development of continuous prawn cell lines for virus isolation and cultivation**

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OBJECTIVES

1. To obtain continuous cell lines from prawn tissues.
2. To select continuous prawn cell lines which are susceptible to virus infection and capable of supporting virus growth.
3. To develop diagnostic procedures for the isolation of viral pathogens (both enzootic and exotic) of prawns using developed cell lines.

NON-TECHNICAL SUMMARY

OUTCOMES ACHIEVED

The major outcome from this project is recognition that routine methods of cell culture are inadequate for the production of continuous prawn cell lines. In addition to obtaining basic information on the physiology and biochemistry of penaeids, utilisation of modern techniques in somatic cell genetics and molecular biology require further investigation.

The aim of the project was to develop continuous cell lines, through the use of mutagenesis of cultured cells derived from prawn tissues, to improve our capacity to isolate and characterise prawn viruses and speed the response to new pathogens that threaten farm production. Thus the cell lines would be susceptible to infection by a range of prawn viruses, such that diagnostic procedures using these cell lines could be developed. In addition, the availability of these cell lines would facilitate the development of other diagnostic procedures (immunoassays and molecular tests) for viral diseases (both exotic and enzootic) of prawns.

Objective 1

A prerequisite for the development of continuous (immortal) cell lines is the establishment of primary (mortal) cell cultures which have a limited life-span but are capable of cellular replication (cell growth and division) over an extended but finite

period. It is during this period of limited cellular division that the primary cell cultures would be amenable to manipulation in such a manner that production and selection of cells with the potential for unlimited cellular replication could be achieved.

In other cell culture systems such as those developed for mammalian or insect cells, nutrient media, supplemented with factors which stimulate growth, have been established for decades. Previous studies have demonstrated that these media can be used to establish primary cultures of cells derived from various prawn tissues but, to date, continuous (immortal) prawn cell lines have not been developed. This project, drawing on previous experience from mammalian and insect systems, was aimed at the development of continuous prawn cell lines. By switching on genes which promoted cell division, or by switching off genes which prohibited cell division, it was anticipated that prawn cell lines could be developed using this mutagenesis approach, as has been achieved with other systems.

Preliminary studies were undertaken to reproduce the establishment of primary prawn cell cultures demonstrated previously by other research groups. These primary cultures would be the starting point for any mutagenesis studies. While the initial aim of establishing primary cultures was achieved it became evident that such cultures had a very restricted potential for cellular division. Indeed, contrary to previous studies, it was concluded that very few, if any, cells in these cultures were actively dividing. This conclusion was supported by results from experiments which directly measured the number of cells in the cell division cycle.

Thus primary cultures derived from prawn post-larval tissues, due to the lack of high levels of cellular replication, were deemed inappropriate for mutagenesis studies. Nevertheless, there were other sources of prawn cells which demonstrated high levels of cell division such as embryonated eggs. In collaboration with AIMS and CSIRO Marine Research, studies were undertaken to establish primary cultures from early stage embryos. While it was demonstrated that embryos could be stripped of their fertilisation membrane to allow access to the cells, the process could not be scaled up to generate sufficient numbers of denuded embryos to establish primary cultures with adequate numbers of cells for further manipulation.

Objectives 2 and 3

Objectives 2 and 3 were dependent on achieving objective 1. While objective 1 was not achieved, results from this project have demonstrated unequivocally that current materials (existing culture media) and methods (tissue explants) are inadequate for the development of continuous prawn cell lines. As with other systems it may be necessary to return to basics i.e. undertake research on the development of a specialised culture medium for prawn cells including the identification of prawn cell growth factors. In addition, utilisation of molecular techniques for manipulating prawn genetic material to facilitate cell line development should be assessed. Thus the findings of this project provides information essential to the establishment of future research projects aimed at the development of continuous prawn cell lines.

While continuous prawn cell lines have not been produced, it is noteworthy that primary cultures have been maintained for over 140 days and will be maintained until they die or undergo spontaneous transformation and initiate cellular replication.

KEYWORDS: prawn cell culture, cell lines, prawn viruses

2.0 ACKNOWLEDGEMENTS

The authors wish to acknowledge the significant contributions of several institutions and individuals without which this project would not have been possible. In particular, the enthusiastic involvement of the Australian Institute of Marine Science (AIMS), Cape Ferguson, Queensland, which provided facilities, prawns, and staff to assist with prawn husbandry during the course of this project, was instrumental. In addition, AIMS staff provided valuable input during discussions on experimental design, and the contributions of Dr Mike Hall, Mr Matt Kenway, Mr Mathew Salmon and Mr Don Booth are greatly appreciated.

CSIRO Marine Research's Cleveland Laboratory, Queensland was also an enthusiastic participant and provided prawns and facilities for this project. Dr Belinda Norris, Dr Peter Crocus and Dr Nigel Preston were particularly helpful.

The authors thank these intitutions for their willing participation and for tolerating disruption of their normal routine by scientists from down south.

3.0 INTRODUCTION

3.1 BACKGROUND

The current lack of continuous (immortal) prawn cell lines suitable for the isolation and cultivation of viral pathogens of prawns is a serious obstacle to effective disease control for the prawn farming industries (Crane and Bernoth, 1996). Viral diseases of prawns have had and continue to have a serious negative impact on the Australian industry. Currently, the Australian prawn farming industry is valued at approximately AUS$50 million and an estimate of the losses through viral disease is placed at approximately 40% mortality. This project will bring together cell culture/aquatic animal disease expertise at AAHL and prawn biology expertise at AIMS and will complement other work currently undertaken. For example, the development of molecular and/or immunodiagnostic tools for virus identification would be greatly facilitated by, if not reliant on, the availability of continuous cell lines suitable for virus cultivation (Mari et al., 1993a, b). This project has been discussed at the Aquaculture CRC meeting held at Brisbane (October 1996) which included scientists and industry participants in prawn health research and by the prawn virus research group. The aim of the project is to develop continuous cell lines, through the use of mutagenesis of prawn tissues, to improve our capacity to isolate and characterise prawn viruses and speed our response to new pathogens that threaten farm production.

The growing occurrence of viral diseases in farmed prawns worldwide, including Australia, has dramatically reduced and continues to threaten production (Lightner et al., 1995; Rosenberry, 1995; Wongteerasupaya et al., 1995). Work carried out to date on crustacean viruses has demonstrated that new viral species are being encountered and that, often, there are mixed infections with more than one virus present (Lightner et al., 1993; Owens, 1993). Thus disease control is extremely difficult; the viruses have to be separated from each other to identify the pathogen, before appropriate disease management regimes can be organised, and before virus characterisation, including molecular probe development, can be undertaken (Lu et al., 1991; Lightner et al., 1993).

The extent to which characterisation of new viruses is slowed by the lack of suitable cell culture techniques has been illustrated by the time scales required to isolate the viruses involved in the relatively recent disease outbreaks in Australia. Work has had to rely upon tests with whole animals and the sources of these cannot be guaranteed virus-free. Prior to the availability of in vitro systems for vertebrates, virologists utilised whole, live animals as their test systems to grow viruses and identify viral pathogens. In the 1950s, development of cultured cell monolayers, particularly continuous cell lines, greatly facilitated virus cultivation, isolation, quantification and characterisation (Luria et al., 1977). Indeed, the basic techniques established then are still in use today (Murray et al., 1995). The capability to grow viruses in continuous cell lines not only facilitates purification of viruses from mixed infections and positive identification of disease-causing agents but also provides a continuous source of purified virus for further studies.

The availability of continuous (immortal) cell lines has several advantages over primary (mortal) cell cultures as a research and diagnostic tool. Firstly, being immortal, cell lines can be continuously passaged in vitro with no need to return to whole animals (prawns) as the source of cells; establishment of primary cell cultures is a tedious, labour-intensive and time-consuming technique. Moreover, continuous

cell lines are clonal, i.e. they are relatively homogeneous with respect to genotype and phenotype with stable properties such as virus susceptibility, and can be stabilised in liquid nitrogen for several years. Continuous cell lines can be mass cultured within a short time period which may be essential in the face of a serious and extensive disease outbreak.

A further limiting factor in the cultivation of viruses is the host- and/or tissue-specificity exhibited by some viruses. In this respect many of the major viral pathogens of finfish require the use of fish cell cultures of specific origin to support their growth and development. The commercial success of the salmonid and cyprinid aquaculture industries world-wide is due, in part, to the development of fish cell lines (Wolf and Mann, 1980) which are used to monitor farmed fish populations for the presence of specific viral pathogens. Even in this era of molecular biology, virus isolation in cell culture, where available, is still considered the most sensitive technique for the detection of viruses (Leong, 1995) and is an integral part of the OIE Aquatic Animal Health Code and Diagnostic Manual for Aquatic Animal Diseases developed to control the spread of diseases through international trade (OIE, 2000, 2001).

The aim of this project, using mutagenesis of prawn tissues (c.f. Trott et al., 1995), is to develop continuous cell lines to improve our capacity to isolate and characterise prawn viruses and speed our response to new pathogens that threaten farm production, bringing together the considerable skills in cell culture available at AAHL and researchers at AIMS who have prawn cell cultures that have been maintained for some time (Fraser and Hall, 1999). In addition, this project would complement the work of Dr. Lani West, QDPI, on primary prawn cell cultures, for which basic culture conditions have been elucidated.

Prior to commencement of this project it was recommended, in order to obtain the most recent data, that an international workshop on prawn cell culture should be convened. It was recognised that since there were few, if any, established (immortal) cell lines derived from prawn tissues reported in the literature, there may be unpublished information from laboratories with a long history of involvement in prawn cell culture research. With support for Aquaculture CRC Ltd., Drs Mark Crane (CSIRO AAHL) and John Benzie (AIMS) organised an international workshop on prawn cell culture to which known experts on prawn, crustacean, molluscan and insect cell culture were invited to participate.

3.2 NEED

The ability to isolate viruses in cell culture is fundamental to disease diagnosis in both human and veterinary (including aquatic animals) medicine. In addition, the ability to grow the virus in culture provides a potentially limitless source of pure virus and thus facilitates further characterisation of the virus and development of more sophisticated and improved diagnostic procedures. At present, virus isolation in cell culture remains, for most pathogenic viruses where cell culture systems have been developed, the most sensitive and reliable technique for the detection of viral pathogens of fish (OIE, 2000).

The current lack of continuous prawn cell lines suitable for the isolation and growth of prawn viruses is a major set-back for the diagnosis of viral diseases of prawns (see

Crane and Bernoth, 1996, for review); isolation and identification of the causative agents is severely hindered and the development of other diagnostic procedures is slowed.

The application of virus isolation in cell culture and the critical role it plays in certifying freedom of disease and controlling the spread of disease is exemplified by its use in the international trade of salmonid products (OIE, 2000, 2001). Individual salmonid cell lines are susceptible to infection by a range of salmonid viruses and provide an essential tool for health surveillance and certification programs and is a requirement for the international trade of specific products. Similar regulations may, in the future, be required for international trade of penaeid products.

The aim of this project is to develop continuous prawn cell lines which are susceptible to infection by a range of prawn viruses, to develop diagnostic procedures using these cell lines and to demonstrate the application of these cell lines to the development of other diagnostic procedures for viral diseases (both exotic and enzootic) of prawns.

3.3 OBJECTIVES

1. To obtain continuous cell lines from prawn tissues.

2. To select continuous prawn cell lines which are susceptible to virus infection and capable of supporting virus growth.

3. To develop diagnostic procedures for the isolation of viral pathogens (both enzootic and exotic) of prawns using developed cell lines.

4.0 METHODS

4.1 Juvenile and Adult Prawns

4.1.1 Source of Prawn Tissues

Various prawn tissues were sourced from juvenile prawns and from spawning adults. Prawns, *Penaeus monodon*, were obtained from the Australian Institute of Marine Science (AIMS), Townsville QLD and *P. japonicus* from CSIRO Marine Research (CMR), Cleveland QLD. Experiments aimed at establishing primary cell cultures from tissues of adult and juvenile prawns were undertaken at the Australian Animal Health Laboratory (AAHL), CSIRO Livestock Industries, Geelong, at AIMS and at CMR.

Prawns used at AAHL, were transported from Queensland to Geelong where they were maintained at AAHL in aerated marine aquaria at a constant temperature of 26^{o}C. Prawns were fed twice daily using fresh squid or commercial pellets. Water quality was monitored daily.

4.1.2 Disinfection of Prawns

On the day prior to obtaining tissues for cell culture, prawns were transferred to a small isolated aquarium, with antibiotics added to water (antibiotic seawater) at the following final concentrations:

Penicillin:	1000 IU/mL
Streptomycin:	1000 µg/mL
Amphotericin B (Fungizone):	5 µg/mL
Gentamycin:	50 µg/mL

Following overnight maintenance in antibiotic seawater, individual prawns were sedated by transferring them to aerated, chilled seawater (which had been maintained in a -70^{o}C freezer overnight) until movement ceased. Only one prawn was manipulated at any one time. Following sedation, in order to disinfect the carapace, prawns were transferred to chilled (maintained at 5-10^{o}C using ice), 10% (w/v) sodium hypochlorite in seawater for 15 minutes. Prawns were removed and the surface wiped with 70% ethanol. At this stage each prawn was dissected and tissues from all major internal organs (heart, hepatopancreas, epidermis, tail muscle, gut, lymphoid organ, eyestalk, antennae, eye, gonad) were obtained and placed in cell culture medium.

In addition to using juvenile prawns as sources of tissues for cell culture, other life-cycle stages were utilised including nauplii.

4.2 Eggs and Nauplii

4.2.1 Source of Eggs and Nauplii

Embryonated eggs and nauplii of *Penaeus monodon*, were obtained from the Australian Institute of Marine Science (AIMS), Townsville QLD and of *Penaeus japonicus* from CSIRO Marine Research (CMR), Cleveland QLD. Experiments aimed at establishing primary cell cultures from these life-cycle stages were undertaken at

the Australian Animal Health Laboratory (AAHL), CSIRO Livestock Industries, Geelong, AIMS and CMR.

4.2.2 Disinfection of Eggs and Nauplii

Disinfection of fertilised eggs and nauplii is necessary to inactivate microbes present in seawater which would contaminate any subsequent primary cell cultures, overgrow and destroy any prawn cells present. Surface disinfection of the eggs, was achieved using a modification of the methods recommended by OIE (2001). The recommended disinfection protocol for eggs is washing in 100ppm formalin for 30 to 60 seconds then in 0.1 ppm iodine for 1 minute followed by water rinsing. Nauplii were disinfected using the same procedure with 400 ppm formalin.

This method was modified by using 1000 ppm formalin followed by 0.1 ppm iodine treatment. The formalin and iodine solutions were prepared in filter-sterilised seawater. Eggs treated using the OIE disinfection method did not yield better cultures than repeated rinsing in double strength antibiotic seawater. Therefore the OIE method was not used for eggs.

4.3 Tissue Dissociation

4.3.1 Tissue

Prawn tissues were dissociated using a range of techniques including:

- Mincing (using dissection instruments)
- Mincing by passing the tissues through a cell sieve (60 mesh size) obtained from *Sigma* (Cat # S1020)
- Homogenisation using various types of tissue homogenisers
- Passage though a syringe and needle (gauge: 18G, 21G)
- Enzyme digestion using 0.25% (w/v) trypsin (1:250) in hepes-buffered saline (hepatopancreas and heart tissue only)
- Mechanical grinding in mortar and pestle with sterile sand
- Aspiration of minced tissue through a 10 mL glass pipette

4.3.2 Nauplii

Attempts to dissociate other life-cycle stages (freshly hatched nauplii, approximately 13 hours post-spawning and up to two days post-spawning) were made using similar methods, as follows:

- Nauplii were surface sterilised using 200 ppm formalin prior to further processing
- Passage through stainless steel sieve (60, 80 and 150 mesh *Sigma* Cat # S1020, S3770 and S4020)
- Disruption of nauplii by treatment with freshwater
- Freshwater treatment followed by passing though a stainless steel sieve (150 mesh)
- 3 and 6 strokes with in a glass dounce homogeniser
- 3 and 6 strokes with a Teflon coated dounce homogeniser

4.3.3 Embryonated Eggs

4.3.3.1 Introduction

Previous studies (Toullec et al., 1996) had shown that cells from early stage embryos could be used for cell culture. Since all cells of early stage embryos are actively proliferating, embryonated eggs were considered an attractive source of prawn cells for establishing primary prawn cell cultures. Experiments with embryonated eggs of *P. monodon* and *P. japonicus* were conducted at AIMS and at CMR in collaboration with staff at those institutes.

Mature, gravid female prawns were induced to spawn by eye-stalk ablation (Hall et al., 2000) and monitored for the release of eggs (spawning), which usually occurred between 11pm and 2am (with the exception of photomanipulated *P japonicus*). Eggs were collected and processed to obtain cells in a sterile state suitable for cell culture.

Spawning monitors, which detect turbidity in the water, were attached to individual tanks containing one female, sounding an alarm when the female spawned. At this stage, eggs are buoyant and a fine mesh sieve was used to collect eggs immediately after spawning. Using 1-µm-filtered seawater, eggs were washed from the sieve into a beaker containing a minimum amount of seawater.

The suspension of eggs was concentrated to a volume, allowing ease-of-handling without compromising their subsequent development. Although females spawned, not all the eggs were fertile and therefore did not always develop. This was not evident until approximately 1 hour post-spawning, when, by microscopic examination, it was observed that eggs had not undergone cell division or had divided asymmetrically.

4.3.3.2 Fertilisation Membrane Removal

Early-stage embryonated prawn eggs contain cells, which are undergoing rapid cell division (Hall et al., 2000) and thus represent a good source of cells for *in vitro* cultivation. Methods to obtain these cells, using physical disruption of the fertilisation membrane, are too harsh and result in cell death (Lynn et al., 1993). Showman and Foerder (1979) demonstrated that the pesticide, 3-amino-1,2,4-triazole (ATA) inhibits hardening of the fertilisation membrane of the sea urchin, *Strongylocentrotus purpuratus*, and subsequent to treatment, the membrane can be removed using pronase or by passing the eggs through a *nitex* mesh without damaging the embryos. Furthermore, the cells of the embryos could be dissociated using Ca^{2+}-free seawater.

In a more recent publication, Lynn et al. (1993) applied this method to embryos of the shrimp, *Sicyonia ingentis*, to allow immunofluorescent staining of internal cellular components. Toullec et al. (1996), extended these studies to embryos of the penaeid prawn, *Penaeus indicus*, and demonstrated that cells obtained in this manner could be used to initiate cell cultures which remained viable for extended periods. Moreover, cells in these cultures demonstrated cell division and differentiation into different cell types, thus demonstrating normal cell function *in vitro*.

Fertilised, embryonated eggs were treated as follows:

Initially experiments were conducted to determine if eggs developed and hatched in antibiotic supplemented sterile seawater as opposed to unsupplemented sterile seawater. There were no detrimental effects using antibiotic seawater.

As soon as possible after collection, eggs were placed into various concentrations of ATA (0.5, 1, 2 and 5 mM). ATA exposure times varied from 2 minutes through to between 3 and 4 hours when the embryos were at the 32-cell stage of development.

After ATA treatment eggs were placed into sterile seawater supplemented with antibiotics and developed to the 16-cell stage when further manipulations were performed to encourage removal of the fertilisation membrane.

Attempts to rupture and/or remove the egg envelopes from ATA-treated eggs, to release cells were undertaken using the following methods:

- Forcing eggs through 210 or 260 µm internal diameter needles
- Pushing eggs through *nitex* mesh (pore sizes 200, 210, 212 or 236 µm) attached to a syringe barrel
- Manually tearing envelopes using corneal forceps
- Sonication
- Vortexing eggs with glass beads
- Cutting eggs/envelopes with a scalpel blade
- Forcing eggs through 18, 21, 22, 23, 25, 27 G needles

4.4 Primary Cell Cultures

Tissues or cells obtained from juvenile prawns, embryonated eggs and nauplii were placed in cell culture medium under various culture conditions. Initially, sixteen types of cell culture media were used as shown in table 1. For example, medium #12 in the table consists of 2 X concentrated Leibovitz's L-15 medium supplemented with 10% (v/v) foetal bovine serum and 10mM pipes buffer. All media contained 2mM glutamine, 50 µg/mL gentamycin and 5 µg/mL amphotericin B.

Table 1. Formulations of Cell Culture Medium Used in Initial Experiments

Supplements	Cell Culture Medium							
	Leibovitz's L-15 medium				Eagle's minimal essential medium			
	1 x conc.		2 x conc.		1 x conc.		2 x conc.	
Foetal Bovine Serum	5%	10%	5%	10%	5%	10%	5%	10%
10mM Hepes buffer	1	2	3	4	5	6	7	8
10mM Pipes buffer	9	10	11	12	13	14	15	16

Initially, an empirical approach, by examination of cultures by light microscopy and using cell morphology and survival as basic parameters, was used to evaluate various cell culture medium formulations. Based on results obtained with these initial sixteen media types, further modifications were used in subsequent experiments. These other cell culture media are shown in table 2.

In addition, following a literature review, a list of cell culture supplements, which appeared to be beneficial to prawn cell cultures in studies by other laboratories, was compiled and these supplements were added to basic medium types used in this study (table 2). In addition, other supplements not previously reported were also trialled. Such supplements included the following:

Chen salts

Chen and Kou (1989) obtained confluent cell cultures derived from the Oka organ of *P. monodon* using a modified 2X L-15 medium which was supplemented with a salt solution containing a mixture of NaCl, KCl, $CaCl_2$, $MgSO_4$ and $MgCl_2$.

Basic Fibroblast Growth Factor (bFGF)

Hsu et al. (1995) demonstrated that cell cultures derived from the oka organ of *P. monodon* and treated with bFGF could be sub-cultured over an extended period of time indicating that the cells were replicating and may have been transformed. Unfortunately, the cultures could not be cultured beyond passage 90. From these studies, it was concluded that L-15 medium supplemented with 5g/L NaCl, 1g/L glucose (Hsu salts), 10% FBS and antibiotics was the best basic nutrient medium.

Serum source

The AAHL tissue culture section has a number of different sera (e.g. horse serum, chicken serum) in stock which were used as supplements in a number of different culture media. In addition, prawn haemolymph obtained from *Penaeus monodon* stocked at AIMS was also used as a supplement to cell culture media.

Thus, as experimentation with primary cell cultures using various cell culture medium formulations proceeded, cellular morphology and ability to form monolayer cultures were used as the criteria to determine optimum culture conditions. In this way it was possible, by a process of elimination, to select the more successful cell culture media.

Seawater modified medium

In addition, to relatively standard cell culture media, other novel media preparations were trialled. One such medium was a seawater modified medium (90% sterile seawater, 10% 2X L-15) supplemented with various additives including prawn haemolymph. Also, a customised medium designed by Dr Lani West, QDPI, was made available to this project.

Osmolarity

The pH and osmolarity of media types (without antibiotic and FBS) was tested using an Orion pH meter and a Wescor (model 5100C) osmometer. For each medium type duplicate 20mL aliquots were measured and the mean calculated.

Culture vessels

Primary cell cultures were established in a variety of culture vessels including 25 cm^2 culture flasks (*Corning*, *Nunc* and *TRP*), Labtek chamber slides (*Nagle Nunc International*), 24-well culture plates (*Linbro*) and 96-well culture plates (*Nunc*).

Table 2. Modified Cell Culture Media Used for Primary Cell Cultures

Medium type	Supplements and concentrations used
1 X EMEM	10mM hepes, 2mM glutamine, 100µg/mL gentamycin and 5µg/mL amphotericin B, 5% or10% FBS
1 X EMEM	10mM hepes, 2mM glutamine, 250µg/mL gentamycin and 5µg/mL amphotericin B, 5% or 10% FBS
1 X L-15	10mM hepes, 2mM glutamine, 100µg/mL gentamycin and 5µg/mL amphotericin B, 5% or 10% FBS
1 X L-15	10mM hepes, 2mM glutamine, 250µg/mL gentamycin and 5µg/mL amphotericin B, 5% or 10% FBS
2 X L-15	10mM hepes, 2mM glutamine, 250µg/mL gentamycin and 5µg/mL amphotericin B, 5% or 10% FBS
2 X L-15	10mM hepes, 2mM glutamine, 250µg/mL gentamycin and 5µg/mL amphotericin B, 5, 10 or 20% FBS and 20ng/mL bFGF
1 X L-15	10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% FBS **or** 10% heat inactivated FBS
2 X L-15	10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% FBS **or** 10% heat inactivated FBS
2 X L-15	10mM pipes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% FBS **or** 10% heat inactivated FBS
1 X L-15 with Hsu salts	5g/L extra sodium chloride, 1g/L glucose,10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% FBS
2 X L-15 with Hsu salts	5g/L extra sodium chloride, 1g/L glucose,10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% FBS
2X L15	1g/Lglucose, 10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% FBS
1X L-15	5g/L extra sodium chloride, 10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% FBS
2 X L-15	5g/L extra sodium chloride, 10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% FBS, 20ng/mL bFGF
2 X L-15	10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% "Trace" FBS
2 X L-15	10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% horse serum
2 X L-15	10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% avian serum
2 X L-15	10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% SPF chicken sera
1X M199	10mM hepes, 2mM glutamine, 100µg/mL gentamycin and 5µg/mL amphotericin B, 5% or 10% FBS
1X M199	5g/L extra sodium chloride,10mM hepes, 2mM glutamine, 100µg/mL gentamycin and 5µg/mL amphotericin B, 5% or 10% FBS
1 X M199 with Chen salts*	5g/L extra sodium chloride, 10mM hepes, 2mM glutamine, 50µg/mL gentamycin and 5µg/mL amphotericin B, 10% heat inactivated FBS
Lani West medium	Medium supplied by Lani West from University of Queensland
Seawater modified medium	90% sterile seawater, 10% 2X L-15, 10% FBS, 50µg/mL gentamycin and 5µg/mL amphotericin B, 5% prawn haemolymph
Seawater modified medium	90% sterile seawater, 10% 2X L-15 (5 g/L extra NaCl), 10% FBS, 50µg/mL gentamycin and 5µg/mL amphotericin B, 5% prawn haemolymph
Seawater modified medium	90% sterile seawater, 10% 2X L-15 (5 g/L extra NaCl), 10% FBS, 50µg/mL gentamycin and 5µg/mL amphotericin B, 20ng/mL bFGF

*Chen salts: I litre medium is supplemented with 22g NaCl, 0.8g KCL, 6g $MgSO_4H_20$, 1.8g $CaCL_2 2H_2O$, 0.1g $NaH_2PO_4 2H_2O$. 0.3g glutamine and 2g lactalbumin hydrolysate

4.5 Insect Cell Cultures

4.5.1 *Aedes albopictus* Cell Line

Insect cell cultures of the *Aedes albopictus* cell line C6/36 (derived from mosquito larvae and originally sourced from Dr Ian Marshall at ANU by AAHL in 1990) were used in an attempt to generate a mutant cell line which was deficient in the salvage pathway enzyme hypoxanthine guanine phosphoribosyl transferase (i.e. the mutant would be designated $HGPRT^-$). Such a mutant cell line could be used in fusion experiments with single cell suspensions of prawn tissues to form hybrid cell lines which, by virtue of the insect cell line would be immortal, and by virtue of the prawn cell features would be susceptible to infection by prawn viruses. In a manner similar to the generation and selection of hybridoma cell lines (Goding, 1983) which are formed by fusing a murine plasmacytoma cell line (e.g. sp2/0) with spleen cells from immunised mice, subsequent selection for the hybrid cells would be undertaken using HAT (hypoxanthine, aminopterin, thymidine) medium (Littlefield, 1964). The mutant insect cell line would not grow in HAT medium due to the $HGPRT^-$ mutation. The prawn cells would not grow in HAT medium due to their inability to replicate continuously. Hybrid cells should be $HGPRT^+$ with the capability of replicating in HAT medium.

Stock cultures (passage 73), grown in Basal Medium Eagle (BME) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM glutamine, non-essential amino acids (1 mL 100x in 100 mL medium), 10 mM hepes, 100 IU/mL penicillin G and 100 µg/mL streptomycin, were used as the parent cultures for the selection of mutant cells.

Cells were sub-cultured in cell culture medium containing 15µg/mL 6-thioguanine (Sigma product # A4882) which is a toxic analogue of the nucleic acid precursor guanine and would be incorporated, by the salvage pathway, into $HGPRT^+$ cells only. Maintenance of cell lines in such medium will select for $HGRPT^-$ mutant cells; only $HGPRT^-$ cells, which synthesize DNA by the *de novo* pathway, would survive in this medium.

4.5.2 Spodopteran Cell Line Sf-21

White spot syndrome virus (or WSS-like viruses) appears to have a broad host range (Flegel, 1997) and will grow in a variety of crustaceans (and maybe even insects). So, given the apparent promiscuity of this virus, attempts to grow it in an insect cell-line that is used for conventional baculovirus cell culture, i.e., *Spodoptera* cells, were undertaken.

The Sf 21 cell line of the fall armyworm (*Spodoptera frugiperda*), derived from pupal ovarian tissue, was originally obtained from Invitrogen. Stock cultures, maintained in SF900 11 cell culture medium supplemented with 1% (v/v) FBS and 4 mM glutamine were used for these experiments.

Inoculum used for infection trials consisted of haemolymph collected on day 5 post-infection from WSSV-infected prawns (see section 3.6) diluted 1:10 in cell culture medium.

Sf-21 cells were cultured in 25 cm^2 flasks. When the cells were 80% confluent, 500 uL of dilute virus inoculum was added to each flask (and 500 µL of tissue culture medium, alone, added to mock-infected cells). Virus was adsorbed for 1.5 hr with gentle rocking. Inoculum was then removed, and 10 mL of fresh cell culture medium was added to each flask.

Cultures were maintained at 27-28^{o}C, and supernatant fluids from cultures were passaged every 3-4 days. An aliquot of cells from each monolayer was retained from each passage of infected and mock-infected cells (passages 1, 2 and 3). Total nucleic acid was extracted from each of these samples, and each extract was examined with a real-time PCR for WSSV (McColl, unpublished). A WSSV-containing plasmid (supplied by Richard Hodgson, CLI, Brisbane) was used to quantitate the PCR.

4.6 White Spot Virus

White spot virus originating in Thailand and kindly donated by Dr Tim Flegel (Mahidol University, Bangkok) was used for all viral studies. The purity and identity of the imported virus was confirmed by polymerase chain reaction (PCR) prior to expansion by experimental infection of penaeid prawns. Haemolymph from infected prawns (*Penaeus japonicus*) was harvested 2-5 days post-infection (p.i.) and stored at -80^{o}C until use. Viability of frozen viral stocks was checked by titration studies in penaeid prawns.

4.7 Cell Proliferation Assay

There are a number of cell proliferation assays developed in recent years (Yu et al., 1992) some of which have been developed into commercial kits. Few, if any, have been used on fish cell lines or cultures of other aquatic species (Mulford et al., 2001). For this project, in order to determine whether prawn cell populations maintained *in vitro* contained any proliferating cells, a commercial kit (*Roche* Cat. No. 1 299 964) was adapted for use, firstly with finfish cell lines grown at relatively low temperatures and then with primary cultures of prawn cells.

By use of this kit, actively replicating cells are identified by an immunocytochemical assay for the detection of 5-bromo-2'-deoxy-uridine (BrdU) incorporated into cellular DNA using a mouse monoclonal antibody (Gratzner, 1982). 5-bromo-2'-deoxy-uridine is an analogue of the DNA precursor thymidine and, when present in the culture medium, is incorporated into DNA of replicating cells during the S (synthesis) phase of the cell cycle. Thus the BrdU-specific monoclonal antibody will bind to BrdU-substituted-DNA which can be localised in the nucleus using immunocytochemical techniques.

The assay was performed as per manufacturer's instructions with some modifications, to make the assay more appropriate for finfish cells and for use with prawn cell cultures.

Finfish cell lines used for the assay, included rainbow trout gonad (RTG-2), epithelioma papulosum cyprini (EPC) and pilchard liver (PL). Finfish cell cultures were grown in 8-well chamber slides (*Nalge Nunc International* Lab-Tek) and processed at

1 or 2 days post-seeding, when the cultures are actively replicating and have reached approximately 70% confluency.

Three steps of the *Roche* method were modified to make the assay more suitable for finfish cell cultures.

- To accommodate finfish cell lines and prawn cell cultures, lower incubation temperatures (15-26^{o}C) were used during the cultivation period and when the cell cultures were incubated (for 30-60 minutes) with BrdU reagent.

- A number of fixatives (ethanol, methanol, formaldehyde, glutaraldehyde) were trialled for use with the aquatic animal cell cultures and the fixative of choice was acetic acid (1 part)/ethanol (3 parts) at room temperature, instead of the recommended ethanol fixative at -20^{o}C. The kit contains reagents, which are compatible with ethanol fixation, but also appears to work well using acetic acid/ethanol fixation.

- On completion of the assay, the cell cultures were counterstained with either Mayer's haematoxylin (*Dako*, Lillie's modification) or Diff-Quik II counterstain (Lab Aids Pty. Ltd, modification of Wrights stain).

BrdU assays using prawn cell cultures, (24 and 48 hours post-culture) derived from lymphoid organ and using RTG-2 cell cultures as positive controls were undertaken. Modifications to the *Roche* method as outlined above were used for the assay on prawn cell cultures.

Briefly, cell cultures were incubated with the BrdU labelling medium for 30-60 minutes, washed three times and fixed at room temperature, as described above. The fixed cultures were then exposed to the working solution containing the anti-BrdU monoclonal (mouse) antibody, as recommended, followed by washing, incubation with anti-mouse-Ig-alkaline phosphatase solution. After further washing, the cultures were exposed to the colour-substrate solution, washed and counterstained prior to mounting for examination by light microscopy.

5.0 DETAILED RESULTS

5.1 Primary Cell Cultures Derived from Prawn Tissues

5.1.1 Culture Medium

Three basic medium types, Eagle's minimum essential medium (EMEM), medium 199 (M199) and Leibovitz L-15 (L-15), were used. 1X and 2X concentrations of these medium types were used and supplemented with various concentrations of foetal bovine serum (FBS) and other supplements (see table 1 and table 2). Upto 50 different media combinations were used for this work.

The medium that, according to the subjective analysis used, supported and maintained cell culture development better than others was 2X L-15 Hsu salts (5 g/L extra sodium chloride and 1g/L glucose) supplemented with 10 mM hepes, 2 mM glutamine, 50 µg/mL gentamycin and 5 µg/mL amphotericin B, 10% FBS. This medium had an osmolarity of 881.5 mosmol/kg and a pH of 7.00 (table 3). These measurements are in agreement with other published data and approximate values for normal seawater (c.f. Huang et al., 1999).

Table 3. Summary of pH and Osmolarity Measurements of Various Formulations of Leibovitz L-15 Medium

Media type	**pH** (mean)	**Osmolarity** (mosmol/kg)
1X L-15	7.71	298
1X L-15 5g/L extra NaCl	7.61	451
2X L-15	7.04	721
2X L-15 5g/L extra NaCL	7.00	881.5

At this stage of the project, cultures were evaluated by subjective criteria using light microscopy – (a) cell morphology and (b) ability of cultures to form monolayers. These two criteria were considered prerequisites for development of replicating cell cultures and subsequent development of immortal cell lines, appropriate for diagnostic tools.

In most cases, microbial contamination did not occur in cultures demonstrating that the disinfection process was efficacious, and subsequent dissection of the prawns did not contaminate the cultured tissues.

All other media, including the more novel types such as the seawater-modified media and the QDPI formulation, were either sub-optimal or did not provide any evident advantage over the standard media types (using the criteria described above). Addition of growth factors (e.g. bFGF) did not promote cell growth and division.

5.1.2 Prawn Tissues

Tissues were aseptically removed from juvenile and adult prawns and the tissue dissociated using various methods (see section 3.3). Tissues used for establishment of primary cell cultures included:

- posterior and anterior ovary
- spermatophore/testes
- heart
- hepatopancreas
- membrane removed from the hepatopancreas
- tail muscle
- epidermis from under the exoskeleton
- eyestalk
- eye
- antenna
- gut
- lymphoid organ
- pleopods

In addition, tissues from post-larvae (pL 80) removed ("explanted" using a scalpel blade) and placed in culture included, eyestalk, gill, hepatopancreas, muscle, pleopods, and internal organs.

Primary cell cultures derived from lymphoid organ and hepatopancreas were the most successful in that cells with fibroblastic cell morphology readily attached to the substrate. Within 24 hours incubation, cells had spread forming numerous colonies of up to a hundred or more fibroblastic cells (figures 1, 2 and 3).

Such primary cell cultures were obtained from lymphoid organ on a regular basis and were maintained for extended periods. On occasions, healthy primary cultures were maintained for approximately 19 days before the cultures showed signs of deterioration. Cultures were incubated at 25^{o}C and have been maintained for over four months but, during this period, there was no evidence of cell replication. In replicating cell cultures of fibroblasts, it is normal to observe rounding of fibroblastic cells which occurs just prior to cell division. At cell division (mitosis), the round mother cell divides to form two smaller, rounded daughter cells which would subsequently spread and grow to form the normal fibroblastic appearance observed during interphase. At no time, in any of the cultures established, was this process observed.

However, some cultures have been maintained, with few medium changes, for over 140 days. In these cultures fibroblast-like cells have remained attached to the plastic substrate and have maintained a fibroblastic morphology. Currently, these cultures are being maintained, and will continue to be maintained, with occasional medium changes. It is possible that any of these cultures may progress beyond this 'crisis point' (see Crane, 1999), transform spontaneously, and initiate cellular replication (c.f. Lynn, 1999).

It was concluded that the apparent increase in cell number reported previously by many researchers may have been due to migration and spreading of cells from explanted tissues rather than to cell replication.

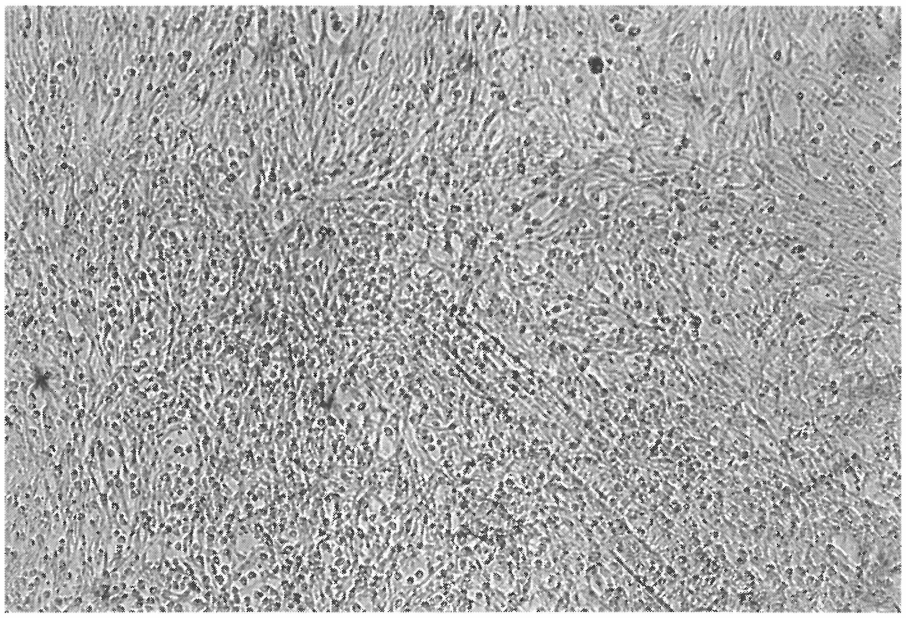

Figure 1. **4-day-old prawn lymphoid organ cells in 96-well plate.** Note the typical fibroblastic appearance of the cells. The cells appear to be healthy and are clearly viable. However, there are no evident signs of cell division (e.g. mitotic figures).

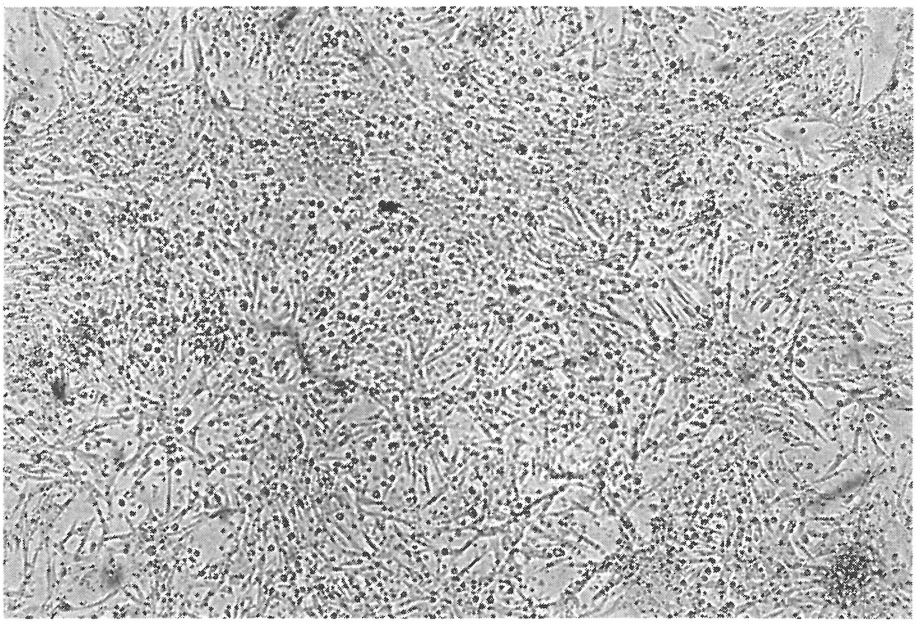

Figure 2. **Photomicrograph of 4-day-old prawn lymphoid organ cells in 24-well plate.** Note the typical fibroblastic appearance of the cells and the similarity of replicate cultures established in 96-well plates (figure 1).

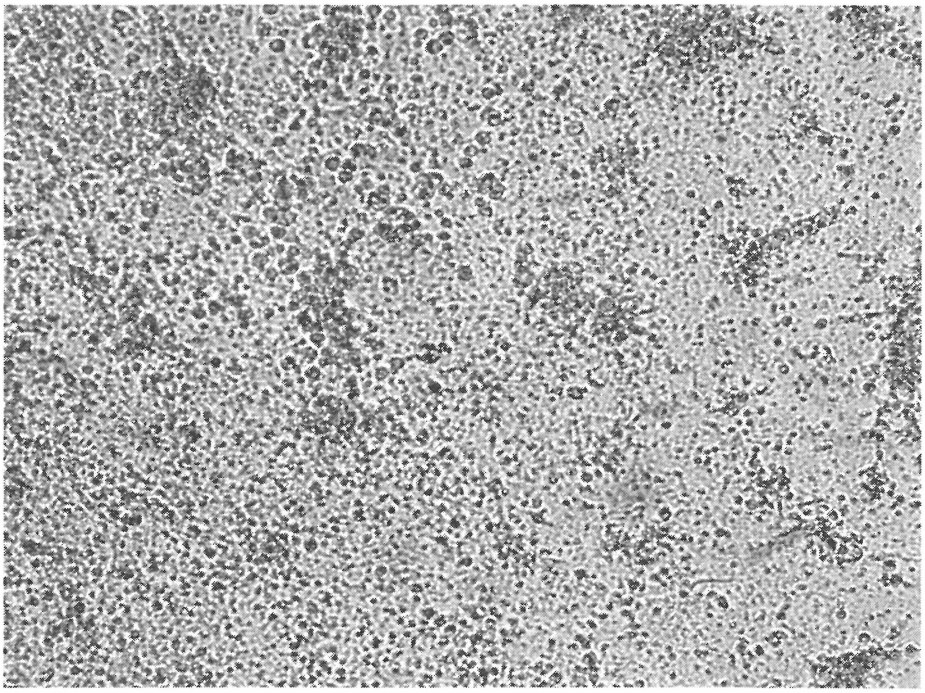

Figure 3. Photomicrograph of 6-day-old prawn lymphoid organ cells in 96-well plate. Note that for many of the cultures at this stage, the cells start to deteriorate, while other cultures can be maintained for prolonged periods without any evidence of cellular replication.

5.2 Disinfection of Eggs and Nauplii

The methods for disinfection (section 3.2.2) were not toxic since subsequent development of disinfected, unprocessed eggs or nauplii occurred as per normal. When disinfected cells and nauplii were incubated at normal temperatures further development occurred resulting in morphological forms indistinguishable from untreated control organisms. In addition, processing of eggs or nauplii through to primary cell cultures usually resulted in no microbial contamination demonstrating that the disinfection process was adequate. Initially, contamination with several species of protozoa was a problem and modification to washing medium (see section 4.4.2) was required to eliminate protozoan contamination from the eggs.

5.3 Nauplii Cell Cultures

Fifteen batches of nauplii (13 hours post-spawning up to approximately 26 hours old) were obtained (in collaboration with AIMS) and various methods were used to disrupt the exo-skeleton to obtain the internal tissue. Following these treatments (see section 3.3.2) processed material was placed in a range of cell culture media (see section 3.4) and monitored for cell attachment and replication. In only a few instances was there any cell attachment observed and in no instances was any cell replication (mitotic figures present followed by and increase in cell number) observed. However, after several attempts to repeat these experiments, it was evident that relatively harsh methods were required to disrupt the nauplii to release internal tissues and that these methods also destroyed the internal organs and cells. Any cells that attached to the substratum did not develop or replicate. As a result no viable cell cultures were obtained using nauplii processed by these methods. It is noteworthy that any undisrupted nauplii remained alive continued to develop in cell culture medium demonstrating that the medium was not toxic to nauplii.

5.4 Primary Cell Cultures derived from Embryonated Eggs

During the first few hours post-spawning, embryonated eggs contain rapidly dividing cells which, if obtained, would be a good source of cells to initiate primary cell cultures (Toullec et al., 1996). Thus eggs were collected during the period just after spawning (6-20 minutes post-spawning), processed (as described in section 3.3.3.2) to remove the fertilisation membrane, and then placed in a range of cell culture media.

5.4.1 Removal of Hatching Membrane

During normal development, the fertilisation membrane is formed around each egg within 12-15 minutes of spawning. This membrane probably acts as a shield against microbial attack and hardens during the egg development process. The membrane is elastic and is difficult to tear or rupture without disrupting the developing cells. Using physical methods in attempts to remove the membrane, the eggs either burst during the processing, rupturing the cells, or the hatching membrane could not be removed and therefore the dividing cells were not released into the growth medium.

Attempts to soften the membrane by treatment with ATA (Toullec et al., 1996) prior to removal were partially successful. However, following treatment of small batches of

eggs it was necessary to use physical methods to rupture and/or remove the membrane.

Eggs were treated with ATA at various concentrations and periods of time with treatment commencing at various times post-spawning (table 4). Once treated with ATA, mechanical methods, which included using *nitex* mesh of various sizes, tearing the envelope using corneal forceps, putting the egg suspension through various gauge needles, sonication, vortexing with glass beads and cutting with a scalpel blade, were used to encourage membrane removal.

None of these methods were very efficient at membrane removal; only a small proportion of eggs had their membrane removed and only a sub-population of the naked eggs appeared to yield healthy cells. Despite treatment with various concentrations of ATA, the hatching membrane was not softened sufficiently to allow the membrane to be removed easily and in doing so release the cells.

Although, cultures were not obtained from eggs with membranes removed, the optimum method appeared to be treatment with 0.5 or 1 mM ATA as early after spawning as possible for the total period up to any further processing (usually to the 16-cell stage), and either putting eggs though 200 µm *Nitex* mesh or a 21 G needle. Manually tearing eggs open using small (corneal) forceps, released cells but these did not survive well in culture.

5.4.2 Protozoan Contamination

Initially, cell cultures were contaminated with various species of protozoa. Therefore, procedures were put in place by which eggs were able to be surface-disinfected after collection to eliminate bacterial and protozoan contamination. Following collection, eggs were surfaced disinfected using either a 100 ppm or a 1000 ppm formaldehyde solution followed by a 0.1 ppm iodophore rinse (OIE method for salmonid egg disinfection), while developing. However, this treatment was detrimental to subsequent egg development and often prevented a majority of eggs from developing normally. Thus this procedure was replaced. By trial and error it was found that rinsing the eggs, while developing, with sterile, 1-µm-filtered, seawater supplemented with 250 µg/mL gentamycin, 5 µg/mL amphotericin B (Fungizone), 500 IU penicillin and 500 µg streptomycin (antibiotic seawater) yielded sterile, healthy eggs.

Immediately after spawning eggs were collected in a sieve and rinsed using the antibiotic seawater into a beaker. Rinses, using an excess of antibiotic seawater, were performed at approximately 20-minute intervals until eggs were developed to the required stage.

5.4.3 Primary Cell Cultures

Treated eggs were placed in culture using a number of different media combinations. These cultures were monitored using light microscopy. Some cultures did contain cells released from eggs which subsequently attached to the substrate and spread (figure 4). However, further development of these cells was not observed. Despite numerous attempts to obtain cultures from embryonated eggs no cultures were viable (yielded replicating cells).

Table 4. Summary of ATA Treatments Used to Remove Fertilisation Membrane

ATA concentration	Treatment time	Approximate time post-spawning eggs added to ATA
0.1mM	10 minutes	10-20 minutes
0.1mM	5 minutes	10-20 minutes
0.1mM	Until processed	10-20 minutes
0.2mM	Until processed	10-20 minutes
0.5mM	Until processed	10-20 minutes
0.5mM	10 minutes	10-20 minutes
0.5mM	5 minutes	10-20 minutes
0.5mM	5 minutes	13 and 19 minutes
0.5mM	10 minutes	12 minutes
0.5mM	10 minutes	13 and 19 minutes
0.5mM	40 minutes (4 cell stage)	6-8 minutes
0.5mM	2 hours 15 minutes (32-64 cell stage)	12 minutes
1mM	Until processed	10-20 minutes
1mM	Approx. 2 hours (16-32 cell stage)	12-14 minutes
1mM	48 minutes	15 minutes
1mM	2 hours 45 minutes (?64 cell stage)	15 minutes
1mM	2 hours 55 minutes	16 minutes
1mM	2 hours 55 minutes	20 minutes
2mM	1 hour 40 minutes	17 minutes
4mM	1 hour 40 minutes	17 minutes
5mM	2 hours 55 minutes	20 minutes

5.4.4 Scale-up of ATA Treatment

The treatment to remove the fertilisation membrane was only partially effective. Thus to increase cell yield, one option was to increase the number of eggs treated. Relatively large batches of eggs could be obtained from *Penaeus monodon* (at AIMS) which were then treated with ATA. However, while it was practical to treat large numbers of eggs in a relatively small volume, the maintenance of eggs under such conditions inhibited their subsequent development. It soon became evident that treatment of large batches of eggs with ATA was not feasible. It is notewothy that the species of prawn used in previous studies (Toullec et al., 1996) was *P. indicus* which may be more amenable than *P. monodon* or *P. japonicus* to ATA-treatment.

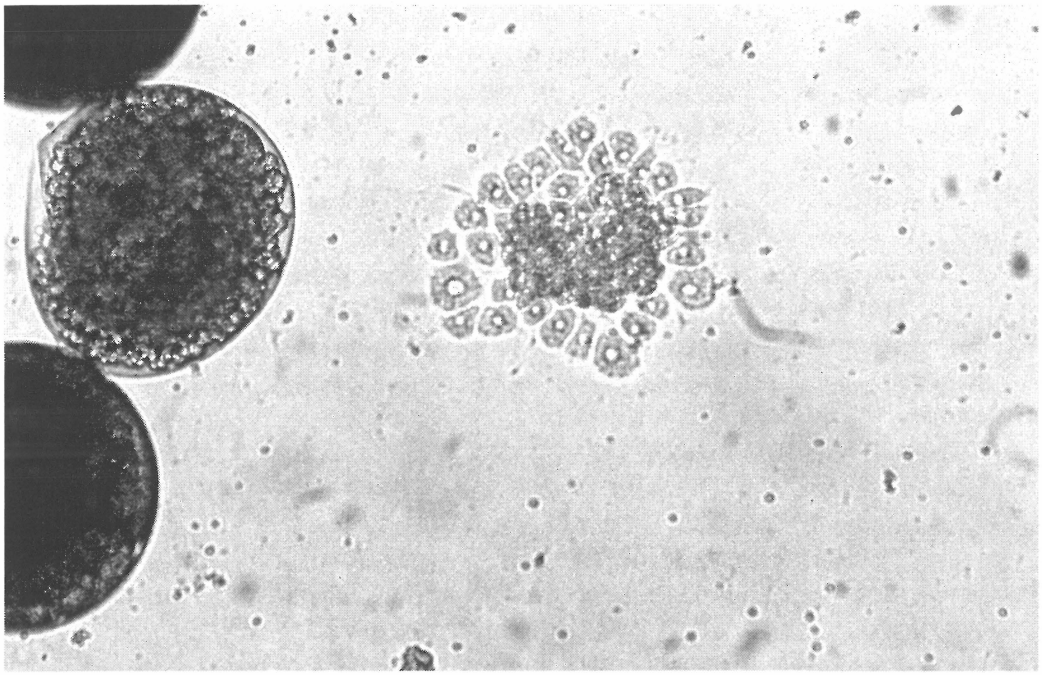

Figure 4. **Vitellin cells from embryonated cells treated with 0.5mM ATA and put through a mesh.** Note that the cells derived from a single embryo have been released and have attached to, and have spread on, the plastic substrate (c.f. Toullec et al., 1996). Other embryos have remained in tact even though their membranes appear weakened by the treatment.

5.5 Insect Cell Cultures

5.5.1 Induction of Thioguanine Resistant Mutants

After several weeks of cultivation in medium supplemented with 6-thioguanine it became evident that the *Aedes albopictus* cell line C6/36 was resistant to this toxic analogue. It is possible that resistance to 6-thioguanine in this cell line is due to low levels or complete absence of the salvage pathway enzyme HGPRTase. To test this hypothesis, C6/36 cells were grown in medium supplemented with HAT. If the C3/36 cell line is indeed $HGPRT^-$ then the cells should die in HAT medium.

5.5.2 Infection of Insect Cell Cultures by White Spot Syndrome Virus

Cell cultures infected with white spot virus inoculum revealed no significant microscopic differences (such as viral cytopathic effect) from mock-infected cultures at first, second or third passage. When nucleic acid extracts of cells from the monolayer of each passage were examined by real-time polymerase chain reaction (PCR), it appeared that there was a large amount of virus present in the first passage, and that subsequent passaging of virus simply diluted the amount of virus that was initially present (figure 5).

The C_T values are shown in table 5 and demonstrate that the amount of virus in each subsequent passage decreased (the C_T values are increasing towards a negative value of 40). Note that the positive control (25 copies of the plasmid/uL) gave a C_T value of 31 indicating that the amount of virus in the first cell passage (C_T value = 23) greatly exceeded 25 copies/µL. The results demonstrate that there was no evidence for replication of WSSV in *Spodoptera* cells in culture.

These results demonstrate the quantitative nature of real-time PCR and the advantage its use has over conventional PCR in obtaining more detailed information about the analysed samples in a relatively easy manner.

Table 5. Summary of Real-Time PCR Analysis of Cell Culture Supernatants from WSSV-inoculated Sp-21 Cell Cultures

Sample	C_T
No template control (negative control)	40
1st passage	23
2nd passage	33
3rd passage	38
Positive control (25 copies/µL)	31

C_T = threshold cycle

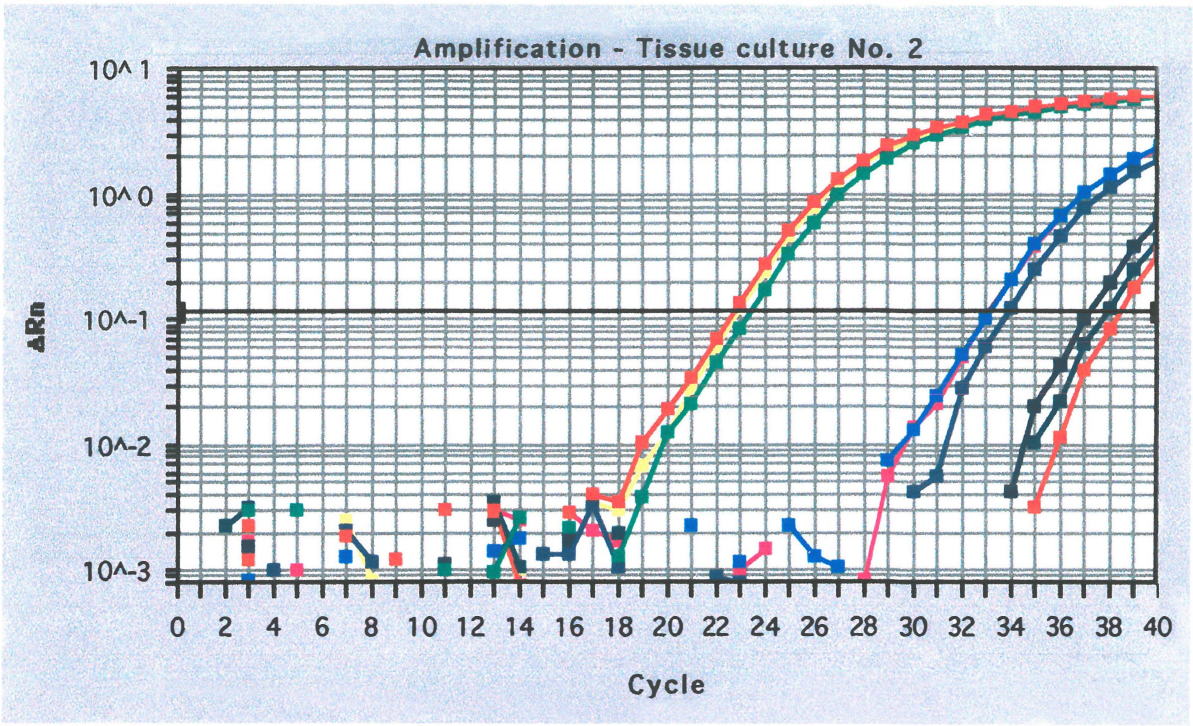

Figure 5. **Reaction curves for the real-time PCRs for each of the samples analysed.** Three samples were analysed, each in triplicate. The red, yellow and green curves represent analysis of the cell culture supernatant from passage 1 culture which contained the highest level of virus (C_T = 23). Passage 2 (pink and blue group) had an intermediate level of virus (C_T = 33) and Passage 3 (blue and red group) had the lowest level of virus indicating that virus was not expanding but actually being diluted as it was being passaged from one culture to the next.

5.6 Cell Proliferation Assay

5.6.1 Fixation of Finfish Cell Cultures for BrdU Incorporation Assay

The *Roche* method (provided with the cell proliferation detection kit) for fixation of cell cultures recommended ethanol as the fixative of choice (due to its compatibility with other reagents) and an incubation temperature of -20^{o}C. For finfish cell cultures, cells detached from the substrate when using this method. Thus alternative fixatives and incubation temperatures were trialled for use with fish cell cultures. The most appropriate method for finfish cell cultures was found to be fixation with acetic acid (1 part)/ethanol (3 parts), at room temperature (approximately 22^{o}C) for 45 minutes. This method (acetic acid/ethanol) resulted in good fixation, with few detached cells, and appeared to be compatible with the other reagents used in the commercial kit. The acetic acid/ethanol fixative was used on various finfish and prawn lymphoid organ cell cultures and repeatedly yielded good fixation and immunocytochemical staining (see below).

5.6.2 Counterstains for BrdU Incorporation into Cell Cultures

In order to facilitate visualisation of the cells a number of different counterstains were evaluated. Two commercially available counterstains were used:

- Mayer's haematoxylin (*Dako*, Lillie's modification)
- Diff-Quik II counterstain (Lab Aids Pty. Ltd, modification of Wright's stain)

For each cell type (various finfish cell lines and prawn cell cultures), duplicate cell cultures were prepared, BrdU-labelled and then treated with one of the two counterstains to determine the most appropriate counterstain for each cell type.

Following the colour reaction and on completion of the washing step, cell cultures were counterstained with haematoxylin for 1 minute, washed with tap water and then a blueing agent, Scott's tap water, added. The cell cultures were then washed again and, after drying, coverslips applied.

On completion of the washing step after the colour reaction, duplicate cell cultures were counterstained with *Diff Quik* added directly to cell cultures, drained and washed in tap water. After drying, coverslips were applied.

Results indicated that counterstaining the cultures made the immunocytochemical labelling easier to interpret. Mayer's haematoxylin appeared to be the better counterstain for the RTG-2 cell line while "Diff-Quik" appeared better for the pilchard liver cell line.

5.6.3 BrdU labelling of Prawn Cell Cultures

Prawn lymphoid organ cell cultures did not appear to have any BrdU labelling when fixed and processed at either 1 or 3 days of culture. Examination by light microscopy did not reveal any labelling, indicating that few, if any, cells were undergoing DNA synthesis at the time of incubation with BrdU. Control cultures of pilchard liver and RTG-2 cells used in the same assay, labelled well (figures 6 and 7). These results indicate that there are very few, if any, replicating cells in the prawn cell cultures.

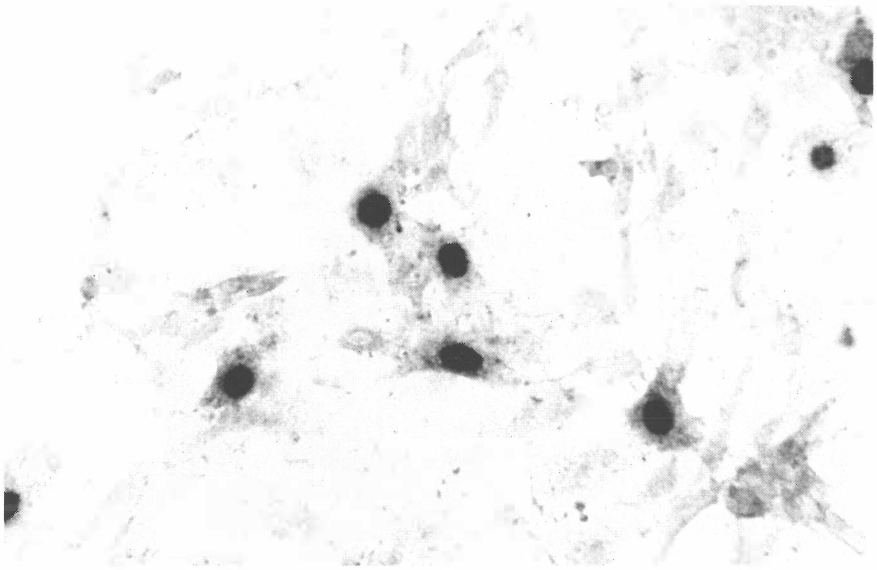

Figure 6. **Photomicrograph of pilchard liver cell cultures labelled with BrdU (400X magnification).** Cells that are undergoing DNA synthesis at the time of labelling with BrdU are positively stained (black nuclei in the photomicrograph) by the immunocytochemical technique carried out on the fixed cell cultures. Unstained nuclei would represent cells in either G_1 (presynthetic) or G_2 (post-synthetic) phases of the cell cycle (Crane, 1999). Stained nuclei were never observed in the prawn cell cultures.

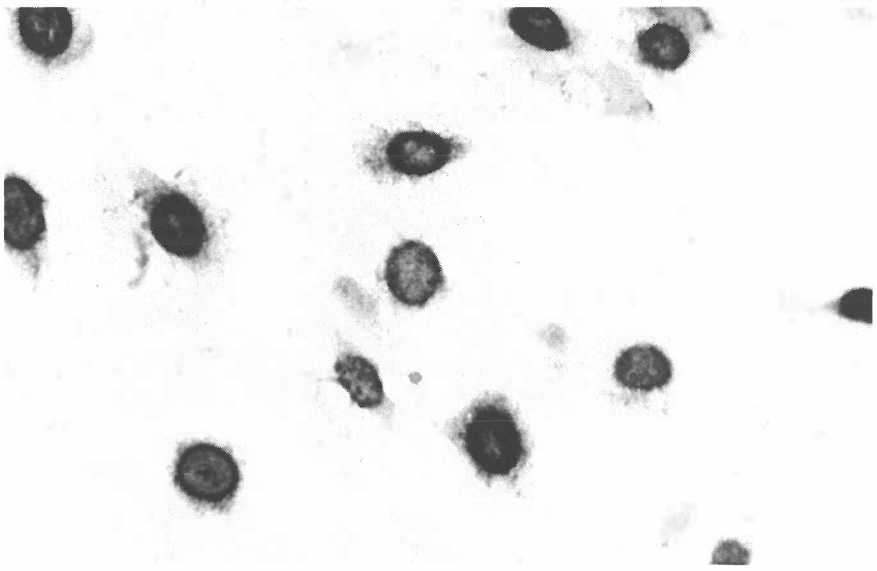

Figure 7. **Photomicrograph of cell cultures of the rainbow trout gonad cell line RTG-2 labelled with BrdU (400X magnification).** Note that there is a higher proportion of labelled nuclei in this cell culture compared to the pilchard cell line. This result correlates with the relative growth rates of the cell lines. The RTG-2 cell line grows at a faster rate than the pilchard cell line. Cell lines with faster growth rates have shorter interphase transit times (usually a shorter G_1 period) and thus there is a higher proportion of cells in S (synthetic) phase at any one time.

6.0 DISCUSSION

6.1 Introduction

The development of continuous (immortal) cell lines from prawn tissues requires the selection of cells which have unlimited potential for cell replication under the culture conditions employed. In most instances, the development of immortal cell lines in other animal species requires cellular transformation (see Crane, 1999 for brief review). The transformation process appears to involve multiple steps controlled by more than one gene and therefore the use of agents, which induce point mutations, is unlikely to be sufficient to induce immortality. It is more likely that prolonged use of relatively low concentrations of mutagenic agents, which are more clastogenic in their mode of action, would induce transformation and allow selection of immortal genetic variants (Trott et al., 1995). It is this concept which formed the basis for this project and the success of this approach is reliant on the use of replicating cell cultures which would be amenable to mutagenesis and selection.

Prior to commencing this project it was recommended that an international workshop be conducted involving experts on prawn cell culture from overseas and Australian laboratories. The outcomes of this workshop would include peer-reviewed publications of relevant research on prawn cell culture, including negative results, which would not normally be published. This workshop, funded by CRC for Aquaculture Ltd, was conducted and provided important information on prawn cell culture. The proceedings of the workshop were published in a special edition of *Methods in Cell Science*.

Several participants at the workshop showed data which indicated that primary cultures of prawn tissues contained proliferating cells (West et al., 1999; Fraser and Hall, 1999) and therefore provided appropriate material for undertaking mutagenesis/transformation studies. In addition, other studies have indicated that at least a small proportion of the cells in primary culture were actively replicating (Ke, 1990; Ellender et al., 1992; Toullec et al., 1996) With this understanding as a starting point, it was agreed that the mutagenesis approach was worthwhile.

However, results from this project showed that, under the culture conditions employed, very few, if any, cells in primary cultures derived from prawn tissues were actively replicating. The use of mutagens without a significant number of replicating cells in the cell cultures would not be effective in inducing and “fixing” any mutations in the genomes of mutagenised cells. DNA synthesis and cell replication is an absolute requirement for fixation of mutations and production of mutant cell lines.

The inability to produce actively replicating primary cultures from prawn tissues could be attributed to any one of several parameters. It is possible that many of the tissues selected for culture had a very limited capacity for cell division. However, the results from the numerous experiments with actively dividing embryonated eggs would suggest that other factors, such as culture medium, were involved. The following discussion addresses some of the issues raised during the course of this project.

6.2 Prawn Tissues

Previous studies by numerous investigators (see Table 6) have demonstrated that primary cell cultures derived from prawn tissues can be established. Tissues which appeared to yield the most successful results included lymphoid organ, ovary and various embryonic tissues. It may be expected that these tissues would have an enhanced potential for cellular replication. However, very few of these studies have demonstrated unequivocally that cells in these cultures were undergoing DNA synthesis and cellular replication. In most studies, it was not specified if there was any attempt to determine whether DNA synthesis or cellular replication occurred in the cultures. In some studies, it was noted that mitosis (cell division) was observed occasionally. Thus, while a range of tissues from various prawns and other crustaceans have been placed in culture, it is difficult to determine whether the tissue source is an important factor to consider when attempting to develop continuous cell lines. No matter what cell culture medium and supplements were employed few, if any, of the tissues (including embryonated eggs) used demonstrated cellular replication. It could be concluded that the cell culture medium used was either deficient in some essential growth factor(s) or that it contained some inhibitor(s) of prawn cellular replication.

6.3 Cell Culture Medium

Based on these studies, and on previous reports by other research groups, 2X L-15 culture medium supplemented with 5 g/L extra sodium chloride, 1g/L glucose, 10 mM hepes, 2 mM glutamine, 50 µg/mL gentamycin and 5 µg/mL amphotericin B and 10% FBS has yielded the best cell cultures to date. This conclusion is based on purely subjective analyses obtained by light microscopic observations. As alluded to, none of the cultures in the present study, nor indeed most any of the cultures in previous studies, demonstrated clear evidence of cellular replication. In order to address this issue many research groups concocted complex media preparations which included tissue extracts from the target species and other supplements used to stimulate cellular replication in other culture systems. It was hoped that such extracts would contain growth factors which would stimulate cellular replication in the primary prawn cell cultures. While it was noted that these supplements may have improved the condition of the cultures, there are no reports describing stimulation of cellular replication (c.f. Hsu et al., 1995).

6.4 Cell Transformation

Cell transformation can be summarised as the process by which primary (mortal) cell cultures acquire certain properties characteristic of neoplastic cells including the ability to replicate indefinitely (c.f. Crane, 1999). There are some preliminary reports of transformed prawn cell lines generated by the addition of exogenous agents. Firstly, Hsu et al. (1995) described transformation of prawn cells using basic fibroblast growth factor resulting in cultures which could be sub-cultured for around 90 passages. Unfortunately, these cultures could not be sub-cultured indefinitely and thus further analysis was not undertaken. During this project attempts to repeat this work were undertaken but were unsuccessful.

Of further interest, Tapay et al. (1995) reported on transformation of primary prawn (*P. stylirostris*) cell cultures by transfecting them with a plasmid containing simian virus-40 tumour (T) antigen gene sequences. The transformed cells were capable of undergoing numerous sub-cultures. To date, it is not known whether the species of origin of these cells has been confirmed, by molecular means, as prawn. Dr P. C. Loh who heads this research group participated in the Aquaculture CRC International Workshop on Invertebrate Cell Culture where he was unwilling to discuss further results with these cultures or entertain any future collaborations using these cells. The current status of these cells is unknown.

These reports indicate that transformation of primary prawn cell cultures to the immortal state should be possible and, together with outcomes of the Aquaculture CRC International Workshop on Invertebrate Cell Culture (Crane and Benzie, 1999), indicate that the mutagenesis approach is feasible. However, results from the present research project indicate that there are few, if any, replicating cells in primary cultures of prawn cells and, under these conditions, transformation is unlikely to occur.

Interestingly, even though we have found no evidence of cellular replication in prawn cell cultures, to date, there are a few cultures which have persisted for over 140 days and these cultures contain relatively healthy looking cells with a fibroblastic morphology. As pointed out by Lynn (1999), at least one insect cell line was established only after ten months of maintenance of quiescent cultures which, after this extended period, initiated replication and subsequently could be sub-cultured within the following two weeks. So far, our experience has been similar and it will be interesting to discover the eventual fate of these long-term prawn cell cultures.

6.5 Conclusions

Numerous attempts to culture cells from prawn (see table 6) and other crustacean tissues (Chang and Brody, 1989; Brody and Chang, 1989) have been undertaken over the last 15 years or so. While these attempts have been very successful in establishing primary cell cultures, very few, if any, have progressed to the development of continuous (immortal) cell lines.

Results from other systems (mammalian, avian, finfish, insect) suggest that development of continuous cell lines for crustaceans should be technically possible. Studies with Syrian hamster (Trott et al., 1995) and human cell cultures (Namba et al., 1988a, 1988b; Tveito et al., 1989; Bai et al., 1993), which have extremely low transformation rates, further support the technical feasibility of obtaining immortal prawn cell lines. However, it should be noted that identification of appropriate culture media and growth factors have been identified in these other systems; the basic culture media for vertebrate and insect cell systems were developed half a century ago and are still in current use (Morgan et al., 1950; Wyatt, 1956; Eagle, 1959; Sachs et al., 1959).

Clearly, from previous studies and from results obtained here, further research on prawn cell cultures is required. There are a number of areas in prawn physiology and biochemistry where basic information is lacking or is currently being obtained which would be of significant benefit to further studies on prawn cell culture and the development of continuous prawn cell lines (see section 8.0).

Table 6. Summary of Recent Studies on Prawn Cell Culture

Species	Tissue	Culture medium	Supplements	Mitosis	Cell proliferation assays	Reference
Penaeus monodon Tiger prawn *P. penicillatus* Red-tail prawn *P. japonicus* Kuruma prawn	Oka organ Ovary Heart Hepatopancreas	2 X L-15	20% FBS Essential amino acids Non-essential amino acids Lobster haemolymph Prawn ovary extract Prawn muscle extract	Not specified	Not specified > 20 days survival	Chen and Wang 1999
P. monodon *P. merguiensis* Banana prawn *Metapenaeus ensis* Greaseyback prawn	Subcutis/epidermis Heart Ovary Lymphoid organ Hepatopancreas Nerve	L-15 2 X L-15 M199 Iscove's MEM	10% FBS Prawn muscle extract Haemolymph Human serum	Very rare	None	Owens and Smith 1999
P. monodon	Ovary	Modified Grace's medium Modified 2 X L-15	5-20% FBS Epidermal Growth Factor Basic Fibroblast Growth Factor Minerals Vitamins Muscle extract	Not specified	3 sub-cultures	Fraser and Hall 1999
P. monodon	Lymphoid organ Ovary	2 X L-15	15% FBS 1% Glucose 5g/L NaCl 15% Muscle extract	Not specified	3 sub-cultures	Kasornchandra et al. 1999
P. japonicus	Lymphoid organ	M-199	20% FBS 0.1 g/L lactalbumin hydrolysate muscle extract Haemolymph additional salts	Not specifies	None	Itami et al. 1999

Species	Tissue	Culture medium	Supplements	Mitosis	Cell proliferation assays	Reference
Nehrops norvegicus Dublin Bay prawn	Haematopoietic tissue	2X L-15 prepared in seawater (25‰ salinity)	10% FBS 5% Nephros serum 5% Nephros muscle extract 0.06 g/L L-proline 1g/L glucose	occasional	negative	Mulford et al. 2001
P. monodon	Haematopoietic tissue Lymphoid organ	Customised medium	Not specified	Observed	1 cub-culture	West et al. 1999
Penaeus indicus Indian white shrimp *P. monodon* *Emerita asiatica* Sand crab	Antennae Eye Eyestalk Gills Muscle	L-15	Not specified	Not specified	Not specified	Purushothaman et al. 1998
P. vannamei White shrimp *P. indicus*	Epidermis Regeneration buds Hepatopancreas Ovaries Embryos	L-15 (#) M199 (!) Grace's medium (*)	10% FBS 0.06 g/L L-proline	Not specified	Not specified	Toullec et al. 1996
P. chinensis Fleshy prawn	Embryos Larvae Eye Heart Lymphoid organ Ovary Cerebral ganglia	2X L-15 (#) Grace's medium (*) MPS	FBS Prawn muscle extract Haemolymph	Not specified	Not specified	Tong and Miao 1996
P. monodon	Oka organ (lymphoid tissue)	L-15	10% FBS 5g/L NaCl 1g/L glucose bFGF	Not specified	Upto 90 passages	Hsu et al. 1995

Species	Tissue	Culture medium	Supplements	Mitosis	Cell proliferation assays	Reference
Macrobrachium rosenbergii Freshwater prawn	Embryos	EMEM L-15 2X L-15 McCoy's 5A medium M199 RPMI-1640 Grace's medium IPL-41 TC-100 Mitsuhashi & Maramorosch medium	5%, 10% or 20% untreated or heat-inactivated FBS 5M NaCl	Not specified	Not specified	Frerichs 1996
P. stylirostris Pacific blue shrimp *P. vannamei*	Oka organ (lymphoid tissue)	2X L-15	20% FBS 4% shrimp extract 30ng/mL epidermal growth factor	Not specified	Not specified	Lu et al. 1995
P. stylirostris *P. vannamei*	Muscle Heart Gill Gut Nerve Oka organ Ovary	2X l-15 M199 2X M199 RPMI-1640 2X RPMI-1640 Grace's medium TC-100 Mitsuhashi & Maramorosch medium	10% or 20% FBS 8% shrimp extract 20 ng/mL NGF or EGF	Not specified	Not specified	Nadala et al. 1993
P. vannamei *P. aztecus* Northern brown shrimp	haemocytes	2X L-15 (#) 2X L-15 (**)	20% FBS	Not specified	Not specified	Ellender et al. 1992
P. stylirostris	Oka organ (lymphoid tissue)	2X L-15	20% FBS 4% shrimp extract 30ng/mL epidermal growth factor	Transformation by transfection with pSV-3 neo. Growth in agarose.		Tapay et al. 1995

Species	Tissue	Culture medium	Supplements	Mitosis	Cell proliferation assays	Reference
P. semisulcatus Green tiger shrimp	Haematopoietic tissue/lymphoid organ Hepatopancreas Ovary	EMEM Ham F-12 L-15 M199	5-20% FBS 5-20% shrimp haemolymph	Not specified	Not specified	Rosenthal and Diamant 1990
P. stylirostris *P. vannamei*	Ovary	M199 L-15 Amphibian culture medium Grace's medium Schneider's medium	10% FBS 2mg/mL proline 2M $MgCl_2$ 5M NaCl	Observed at low rates	Not specified	Luedeman and Lightner 1992
P. orientalis Oriental shrimp	Hepatopancreas	M199	15% FBS	Not specified	17-28 passages	Ke 1990
P. japonicus	Lymphoid organ	RPMI-1640 L-15 2X l-15 EMEM M199 CMRL 1066	18% calf serum shrimp muscle extract haemolymph modified Chen's salts (#)	Not specified	Not specified	Itami et al. 1989
P. monodon	Haematopoietic tissue Ovary	2X L-15	5-20% FBS shrimp muscle extract	Not specified	Not specified	Chen et al. 1988
P. monodon	Gill Gonad Nerve Muscle Heart Hepatopancreas	EMEM L-15 2X l-15 NCTC 135 Various insect media Amphibian medium	10-20% FBS Haemolymph Horse serum Shrimp muscle extract 6g/L NaCl	Not specified	Up to 4 passages	Chen et al. 1986

Chen's salts (Chen, S. N. and Kou, G. H. 1989)
! Itami salts (Itami et al. 1989)
* Luedman and Lightner salts (Luedeman and Lightner 1992)
** Najafabadi salts (unpublished)

7.0 BENEFITS

Principal beneficiary of the development of immortal prawn cell lines, should it occur, would be the prawn aquaculture industry, while the capture fisheries would also accrue some benefit The ability to isolate pathogenic viruses in cell culture will allow a more rapid identification of viral pathogens and provide researchers with better tools to develop improved (more rapid, specific and sensitive) diagnostic procedures. Cell cultures would provide the means to isolate viruses from covertly infected prawns, i.e. from those prawns which are infected but do not show signs of disease. Thus potential brood-stock caught from the wild could be screened for existing viral infections, imported prawns could be screened for the presence of exotic viruses, and on-going aquaculture activities could participate in a more intensive health surveillance program. All these activities are aimed at prevention or control of viral diseases such as those which have decimated prawn industries overseas, costing the industry hundreds of millions of dollars in recent years (Rosenberry, 1995). Unfortunately, we are not at the stage where immortal prawn cell lines can be made available but, due to their importance, further research aimed at their development should be continued.

Thus results from this research project will be of benefit to other groups undertaking studies on the development of continuous prawn cell lines. The application of cellular proliferation assays to cultures of finfish and prawn cells is a novel development which should prove useful in future research. In addition, these studies have demonstrated that further information on the physiology and biochemistry of prawns may be required before prawn cell lines can be developed.

8.0 INTELLECTUAL PROPERTY

No intellectual property (commercially significant developments, patents applied for or granted, licences, etc.) has been generated by this project.

9.0 FURTHER DEVELOPMENT

One of the outcomes of this project has been the identification of specific areas for further research which would benefit future studies aimed at the development of continuous prawn cell lines. These areas of research have been alluded to in previous publications (e.g. Crane and Benzie, 1999) and include:

- Identification of those prawn tissues which, under normal conditions, retain the potential for cell division
- Analysis of prawn haemolymph to aid the development of specific cell culture media for prawn cells (Huang et al., 1999)
- Identification of growth factors for prawn cells
- Transformation by chemical mutagenesis of actively replicating prawn cell cultures (as discussed in this report)
- Transformation by viral oncogenes requires further examination
- The use of somatic cell genetics (e.g. cell fusion systems) to facilitate cell line development.

10.0 STAFF

Apart from the co-principal investigators, officers from State and Commonwealth agencies and representatives from various aquaculture and fisheries industries have been involved in this project and are listed below.

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11.0 DISTRIBUTION

Copies of the final report will be distributed to the following organisations.

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