



Determination of the disease status of Western Australian commercial prawn stocks

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The Fisheries Research and Development Corporation plans, invests in and manages fisheries research and development throughout Australia. It is a federal statutory authority jointly funded by the Australian Government and the fishing industry.

GLOSSARY OF ACRONYMS

AAHL	Australian Animal Health Laboratory - Geelong
bp	base pairs (a measure of length of DNA)
BMNV	Baculoviral Midgut Gland Necrosis Virus
CCEAD	Consultative Committee on Emergency Animal Diseases
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribose nucleic acid
DMSO	Dimethylsulphoxide
EDTA	Ethylene Diamine Tetra Acetic Acid
FRDC	Fisheries Research and Development Corporation
GAV	Gill associated virus
HPV	Hepatopancreatic parvo-virus
IHHNV	Infectious Hypodermal and Haematopoietic Necrosis Virus
ISH	<i>In-situ</i> hybridization
LOV	Lymphoid organ virus
MBV	Monodon baculovirus
MCMS	Mid Crop Mortality Syndrome
MoV	Mourilyan virus
NBF	Neutral buffered formalin
NTU	Northern Territory University
OIE	Office International des Épizooties
PCR	Polymerase Chain Reaction (a method of copying DNA)
PL's	Post-larvae (of prawns and shrimps)
RNA	Ribonucleic acid
SMV	Spawner Mortality Virus
TAFE	Technical and Further Education
TEM	Transmission Electron Microscopy
WSSV	White Spot Syndrome Virus
YHV	Yellow Head Virus

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OBJECTIVES:

1. Detect and document the serious diseases and significant pathogens of wild penaeids in Western Australia.
2. Develop a database of disease, location and prevalence that can assist both government and industry in making informed decisions about translocation of stock.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

- 1) The project has acquired information on the disease status of the commercial prawn species on the northwest shelf and Shark Bay. The information collected indicates that the prawns are free of GAV but are exposed to MBV-like virus and HPV (these two viruses are already known from Australia).
- 2) These data are of use in documenting Australia's prawn disease status both for import and export purposes.
- 3) The project has not negated the hypothesis that the prawn stocks to the west of the Torres Strait have a distinctive parasite fauna and thus controls on movement of prawns between Western Australia and other States and the Northern Territory should either remain or be strengthened.
- 4) The project has provided a greater understanding of the histopathology of prawns in Australia. Syndromes and lesions encountered during this study are described in the report.
- 5) The project has resulted in the development of closer ties between the Fish Health laboratory in Western Australia and the CSIRO.
- 6) Freedom from GAV by Western Australian sourced *Penaeus monodon* is of great scientific interest in that this species is affected by a number of viruses in combination with GAV in both Queensland and New South Wales. The existence of a GAV-free stock may be of use to better understand the role of GAV in Mid Crop Mortality Syndrome (MCMS) and also may assist in attempts to close the life-cycle of *P. monodon* in Australia.

There is little published information on the disease status of the prawns on the north-west shelf, yet these prawns (*Fenneropenaeus merguensis*, *Metapenaeus endeavouri*, *Penaeus esculentus* and *Melicertus latisulcatus*) form the basis of a commercial fishery worth in excess of A\$42 million in 2001-2002. There are also stocks of *P. monodon* on the shelf which form an important source of broodstock for the developing aquaculture industry in Western Australia, and potentially also for the Northern Territory and Queensland.

Unfortunately, prawns are infected with a variety of viral diseases, many of which have been translocated to new areas with movements of the host prawn – mainly for aquaculture but in some cases through frozen product destined for human consumption.

There is a zoogeographic barrier at the Torres Strait so there is no reason to expect that the prawns in Queensland and New South Wales will have the same diseases as those in Western Australia. This is particularly so since the northwest shelf has had little, if any, exposure to other areas through translocations. This presents a unique opportunity to study the viruses and other diseases that may have co-evolved in the area with the prawns. This isolation is already under threat, with, for example, the movement for aquaculture purposes of Gill Associated Virus (GAV) infected post-larvae from Queensland into the Northern Territory.

Thus, there are two disease risks for which this project provides background data. The first is the importation into Western Australia of prawns from other states and from the Northern Territory. To assess adequately the disease risk posed by the imports, we need to understand the local disease status and this has been achieved. The second risk is that diseases endemic in Western Australia may pose a risk to aquaculture establishments in other States. This report provides a basis on which those states can assess the risk to their own industries.

During the five years of the project over 2500 prawns have been examined for disease, mainly by histology, but also by molecular techniques for White Spot Syndrome, Yellow Head Virus, and Gill Associated Virus. Most of the prawns were sourced from the wild fishery, but both *P. monodon* and *P. esculentus* are now being spawned and on-grown in Western Australia under pilot scale or commercial conditions. The disease investigations associated with these nursery or grow-out operations have also been used in compiling this report. The two problems so far encountered in these prawns under aquaculture conditions are Monodon baculovirus-like virus (MBV-like virus) (in *P. esculentus* only) and bacterial problems (both species).

Overall, Western Australian prawns are exposed to MBV-like virus and Hepatopancreatic parvo-virus (HPV). Based on limited electron microscopy, an eosinophilic virus-like inclusion in epithelial cells particularly in the midgut, and similar to HPV but with different staining characteristics, may be a fixation artefact. There are a number of syndromes that may be associated with un-recognised viruses but could equally be due to autolysis and fixation artefacts. Further work is clearly required on these. There is also a rich fauna of metazoan parasites in most of the wild prawns. These are of little concern for aquaculture but probably deserve some taxonomic attention. The lack of MBV-like virus and HPV in *P. monodon* and the absence of GAV in any species are of particular note.

KEYWORDS:

aquaculture, disease, parasites, prawns, translocation, virus.

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CHAPTER 1: GENERAL INTRODUCTION

Background

Little has been published on the actual or potential viral pathogens of prawns in Western Australia, yet prawns (*Penaeus esculentus*, *Melicertus latisulcatus*, *Fenneropenaeus merguensis*, and *Metapenaeus endeavouri*) form the basis for a commercial fishery that is important to the State and the Commonwealth. There is also a by-catch of *Penaeus monodon* in the Nickol Bay area and the prawns appear to be adapted to high-salinity conditions. These *P. monodon* stocks will be an important source of broodstock for the developing prawn aquaculture industry in Western Australia. Prawns of the genus *Penaeus* (*sensu lato*) are of prime importance in prawn culture worldwide, and there is growing interest in farming *P. monodon* in full strength seawater rather than under the estuarine conditions normal in Asia (Sorgeloos 1995; Al-Thobaiti & James 1996).

Unfortunately, penaeid prawns may be infected with one or more of at least 10 viral diseases. These viruses were originally limited in their host spectrum and geographical distribution, but large-scale international movements of penaeids that have occurred over the last 25 years, particularly in the development of penaeid farming in Central and South America, have made many penaeid viral infections almost ubiquitous (Lightner *et al.* 1992a,b).

Disease has had a major impact on prawn aquaculture, particularly in Asia. For example, in 1989 the East Java Province in Indonesia was producing 20,000 tonnes of penaeid prawns per annum. Monodon baculovirus (MBV) was introduced into East Java in 1992/93 and it devastated the industry. Of the 59 hatcheries producing post-larvae in the Situbondo area, only about 50% were again in production by March 1995 (unpublished data, East Java Fisheries Service). More recently White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV) have become a major threat to farm viability and in Thailand these viral diseases are estimated to have caused losses of between US\$400 million and \$1 billion between 1994 and 1997 (Chanratchakool 2000).

In 1995 the virus disease "Taura syndrome", which kills 80%-90% of infected prawns, spread from Ecuador to the USA, probably through the movement of infected *P. vannamei* post-larvae and broodstock (Johnson 1995). Taura Syndrome Virus was subsequently introduced with illegal shipments of *P. vannamei* from the Americas to Indonesia in 2002. Prawn viruses are not just spread by movements of stock for aquaculture purposes. WSSV is believed to have spread to the USA in raw and frozen prawns reprocessed in the USA (Lightner *et al.* 1997).

Australia also has prawn disease issues. Historical records were reviewed by Owens (1997) and known viruses as at January 2002 are summarized in Table 1.1. It should be noted that "Mid-Crop Mortality Syndrome" (MCMS), which was long suspected to be of viral aetiology also occurred in penaeid hatcheries in northern Queensland (Owens 1997). The primary pathogen appears to be Gill Associated Virus (GAV) which is closely related to YHV (Spann & Lester 1997; Spann *et al.* 1997, Cowley *et al.* 2000; Tang *et al.* 2002). Other viruses, including the problematic spawner mortality virus (SMV), are probably involved in MCMS (Anderson and Owens 2001). Almost all of the information on Australian viruses has come from Queensland as a result of the interest in prawn culture there. Prawn parasites and some

prawns recognise the Torres Strait zoogeographic barrier (Owens 1990) so when this project began there was no reason to expect that the prawn viruses described from the east of the barrier would occur in Western Australia and this has proved to be the case.

This project sought to obtain factual data on the disease status of the commercially fished prawn stocks on the north-west shelf of Western Australia, and to define risks to the commercial fishery associated with the translocation of prawns for aquaculture purposes. In aquaculture there is interest in being able to buy post-larvae from Queensland for on-growing in WA, and for buying *P. monodon* broodstock from WA for use in Queensland hatcheries. There is, therefore, considerable risk of disease introduction from Queensland and it is essential to document endemic viruses before translocations occur.

Need

Information on the diseases of prawns across the northwest coast of Australia is primarily needed to reinforce translocation policies in Western Australia, Northern Territory and Queensland and secondly to protect the disease status of the wild stock fishery, in Western Australia worth about \$41 million in 2001-2002 (Dept. of Fisheries State of the Fisheries Report 2001/2002).

Western Australia has approved a number of applications to farm prawns in the north of the state and there is increasing pressure to import post-larvae from Queensland and the Northern Territory, and also to export *P. monodon* broodstock and post-larvae to Queensland and New South Wales. Whether aquaculture of prawns in Western Australia is economically viable or not the translocation of prawns from eastern jurisdictions (and *vice versa*) will result in the introduction of diseases with the potential to affect both the disease status of the State and impact on the wild fishery.

The disease risks are two-fold: from introduced diseases; and from endemic diseases that may be amplified through aquaculture hatchery and grow-out operations before being exported from the State. Both situations can pose increased risks to wild stocks in adjacent waters and to the aquaculture ventures themselves. However, the Department of Fisheries does not wish to hamper the development of farms by imposing unnecessary restrictions on translocations and further, under international trade rules, movements can only be prevented on disease grounds if the disease status of the importing country has been, or is being, determined.

TABLE 1.1. Prawn viruses reported to occur in Australian prawns as at January 2002. Published records only. Abbreviations of viruses follow international practice and are included in the glossary.

Host species	Virus	References	Notes
<i>Penaeus monodon</i>	IHHNV-like	Owens & Hall-Mendelin 1990a Owens <i>et al.</i> 1991	This may be LPV (Owens 1997). Does not react to DiagXotics IHHNV ISH (Anderson & Owens 2001)
	lymphoid parvovirus (LPV)	Owens <i>et al.</i> 1991	
	haemocytic rod-shaped virus	Owens 1993	Similar to WSSV? (Owens 1997)
	MBV-like virus	Lester <i>et al.</i> 1987; Doubrovsky <i>et al.</i> 1988; Vickers <i>et al.</i> 1993, Vickers <i>et al.</i> 2000	Reacts +ve to DiagXotics MBV ISH kits (Anderson & Owens 2001; Spann & Lester 1996)
	GAV/LOV	Spann <i>et al.</i> 1995; Spann & Lester 1997, Spann <i>et al.</i> 2000	Reacts to YHV probe (Tang <i>et al.</i> 2002). YHV and GAV/LOV are distinct viruses (Cowley <i>et al.</i> 1999)
	SMV	Fraser & Owens 1996; Owens <i>et al.</i> 1998	
	HPV	Owens 1997; Munday & Owens 1998	-ve on DiagXotics HPV ISH (Anderson & Owens 2001)
	BMNV-like	Lightner 1996	
<i>P. monodon</i> x <i>P. esculentus</i>	IHHNV - like	Owens <i>et al.</i> 1992b; Munday & Owens 1998	Reacted to IHHNV ELISA (Owens <i>et al.</i> 1992b)
	LPV	Owens <i>et al.</i> 1992	
	haemocytic rod-shaped virus	Owens 1997; Munday & Owens 1998	
<i>Melicertus latisulcatus</i>	MBV-like	Lester <i>et al.</i> 1987; Doubrovsky <i>et al.</i> 1988	
	BMNV-like	Lightner 1996	
<i>P. esculentus</i>	HPV-like	Roubal <i>et al.</i> 1989	
	LPV		
	haemocytic rod-shaped virus	Owens <i>et al.</i> 1993	
	MBV	Lester <i>et al.</i> 1989	
	GAV	Spann <i>et al.</i> 2000	Experimental infection
<i>Metapenaeus bennettiae</i>	Benettae baculovirus	Spann & Lester 1996, 1997	Similar to MBV but doesn't react to MBV probe (Spann & Lester 1996)
<i>Marsupenaeus japonicus</i>	parvo-like virus	Spann & Lester 1997	Smaller virus than HPV
	GAV	Spann <i>et al.</i> 2000	Experimental infection
<i>Fenneropenaeus merguensis</i>	HPV-like	Owens <i>et al.</i> 1988; Owens & Hall-Mendelin 1990b Roubal <i>et al.</i> 1989	Reacts +ve to DiagXotics HPV ISH after modification (Anderson & Owens 2001). Reacts to Asian gene probe for HPV (Lightner 1996)
	LPV	Owens <i>et al.</i> 1991	
	MBV-like	Doubrovsky <i>et al.</i> 1988	
	GAV	Spann <i>et al.</i> 2000	Experimental infection
	haemocytic rod-shaped virus	Owens <i>et al.</i> 1993	

Stocks of prawns off the north coast of WA have not yet been exposed to introductions of prawns from other States or from overseas. They are, therefore, of great value commercially both for the production of specific pathogen free and high health broodstock. This potential market is put at risk by the importation of diseased prawn post-larvae for on-growing. Though steps are taken to minimise the risk of inadvertent spread of diseases to the local wild populations, overseas experience has shown that the disease spread eventually occurs. We also know very little about the existing viruses and their impact on the wild fishery and the relationship to viruses elsewhere in Australia.

Objectives

In order to address this situation, a comprehensive study of wild-harvested and farmed penaeids commenced in 1998 to determine the occurrence, prevalence and distribution of pathogens, parasites and diseases of prawns along the northwest shelf of Western Australia. The study, funded by the Fisheries Research and Development Corporation had two objectives:

- 1) To detect and document the serious diseases and significant pathogens of wild penaeids in Western Australia, and
2. To develop a database of disease, location and prevalence that can assist both government and industry in making informed decisions about translocation of stock.

Report format

It is necessary to have an understanding of the cellular defence mechanisms in prawns to be able to understand the significance of observed histopathology, for example ectopic spheroids. These defence mechanisms are reviewed in the next chapter. The report then details the methodology used (chapter 3). Specific and non-specific histopathological changes observed in the tissues of the sampled prawns are described in chapter 4. The nature, occurrence, prevalence and distribution of microbial, protozoan and metazoan agents infecting penaeid prawns in Western Australia together with a discussion on the regional differences in distribution of infectious agents and the basis on which pathogens parasites and diseases exotic to Australian prawns may be determined, are discussed in chapter 5.

Testing for White Spot Syndrome Virus in Western Australia

Between the end of November 2000 and beginning of December 2000, PCR signals consistent with traces of white spot syndrome virus (WSSV) DNA were detected in Darwin (Northern Territory). The positive signals were obtained from imported prawns (3 of 6 tested) used to feed mud crabs cultured at the Darwin Aquaculture Centre and *Penaeus monodon* prawns cultured at Northern Territory University (NTU), Darwin Aquaculture School. Positive signals were also obtained from mud crabs cultured at the Darwin Aquaculture Centre (2 of 15 tested) and *P. monodon* prawns cultured at NTU (11 of 11 tested). Samples of both shore crabs (5 of 12 tested) and prawns (2 of 4 tested) collected at the outfall of the Darwin Aquaculture Centre were also positive.

A meeting of the Consultative Committee on Emergency Animal Diseases (CCEAD) comprising all State Fisheries Chief Executive Officers and all Chief Veterinary Officers was therefore convened on 6 December 2000 to discuss these findings. Following from this meeting, a national survey was undertaken to test for the presence of the virus. For Western Australia, the collection and testing of prawns, particularly *P. monodon*, was integrated with collecting for this FRDC project and the methods, results and discussion of the Western Australian component of that national survey have therefore been incorporated into this report.

CHAPTER 2: PRAWN CELLULAR DEFENSE MECHANISMS

Overview

Interpretation of histopathological changes observed in diseased prawns depends on a sound understanding of the cellular defence mechanisms and the cellular responses to infectious and non-infectious diseases in prawns.

Understanding of the internal defence mechanisms of crustaceans began in the 1880's through the pioneering work of Metchnikoff on phagocytosis and the inflammatory process. Likewise, Cantacuzène, in a series of papers between 1912 and 1934 pioneered the ongoing investigation of humoral defence responses. Work then languished until the 1960's (Sinderman 1971) when interest was renewed as research began on crustacean diseases of major economic significance, particularly *Gaffkaemia* in lobsters and fungal infections of freshwater crayfish.

Cellular defence mechanisms in prawns can be divided, for convenience, into three broad groupings: maintenance of exoskeleton integrity; foreign agent recognition, inactivation and elimination from the internal organs; and repair of damage by toxins. These systems are not mutually exclusive but share five basic processes: phagocytosis; haemocytosis; degranulation; coagulation (clotting); and encapsulation. Cellular defence mechanisms are particularly dependent on circulating haemocytes and fixed phagocytes.

Maintenance of exoskeletal integrity

The chitinous exoskeleton of prawns is an effective barrier that prevents the entry of infectious agents as well as providing muscle anchorage and protecting underlying soft tissue. The first barrier presented by the exoskeleton against invasion is the very thin proteolipid epicuticular membrane or 'surface waxy layer' (Unestam 1973; Malloy 1978; Fisher 1988). Beneath this layer is the calcified exocuticle. This is very difficult to penetrate, even for disease agents secreting extracellular chitinases. By contrast, the soft non-calcified endocuticle is easily penetrated by such agents (Unestam 1973). Maintenance of the epicuticle membrane is dependent on diet (Fisher *et al.* 1976) and it is probable that penetration of this layer by disease agents is also related to the nutritional and moult status of the animal. "Shell diseases" (a generic term given to the results of penetration of the exocuticle by bacterial and fungal agents) are a characteristic of crustaceans held in captivity (Stewart 1993; Owens *et al.* 1992a) and as such could not be expected to feature in a survey primarily of trawl-caught prawns.

Rapid sealing of wounds to the exoskeleton is required to prevent loss of haemolymph and minimize opportunistic invasion. Reactions leading to wound repair in prawns consist of rapid haemocyte accumulation and aggregation at the wound site followed by focal intravascular clotting. Clotting is initiated by contact of hyalinocytes with seawater (Hose & Martin 1989). The clot results from direct conversion of a soluble fibrinogen (coagulogen) into crosslinked fibrin through the action of a coagulin released by haemocyte rupture (Fuller & Doolittle 1971a,b; Durliat & Vranckx 1981; Ghidalia *et al.* 1981; Hose *et al.* 1990; Aono & Mori 1996). This is followed by melanisation of the wound area to form a dense black membrane beneath which the new epidermis forms. Melanin is produced by the action of the enzyme phenoloxidase on melanin precursors (Unestam & Nylund 1972; Bauchau 1981) and has

antimicrobial properties (Nyhlen & Unestam 1980; Söderhäll & Ajaxon 1982). The epidermis involutes into the wound, utilizing the haemocyte network as basal support. New cuticle is formed by this epidermal layer and lies beneath the melanin membrane (Fontaine 1975). In association with the haemocyte response a dense network of collagen-like fibres forms. This fibrous tissue is not resorbed but remains as a scar (Fontaine & Lightner 1975).

Foreign agent recognition, inactivation and elimination

Foreign agent recognition, inactivation and elimination are effected through both cellular and humoral host defence responses. Immunorecognition is thought to be mediated in the haemolymph through recognition molecules including a α -1,3-glucan-binding protein and lipopolysaccharide-binding proteins (). When activated these trigger activation of the prophenoloxidase system – a cascade of serine proteases and prophenoloxidase which in-turn initiates melanization (Söderhäll & Smith 1986; Söderhäll *et al.* 1996). Subsequent host defence responses comprise cellular mechanisms together with humoral responses involving clotting, phagocytic action mediated by lectins and opsonins, circulating antibacterial factors and release of other immunologically active molecules. Invertebrates have not been shown to exhibit acquired immunity (Roch 1999) although proteins with domains belonging to the immunoglobulin superfamily have been demonstrated (Lanz Mendoza & Faye 1996). Humoral responses are outside the scope of this chapter and will not be discussed further.

The relative importance of cellular and humoral host defence mechanisms in prawns has yet to be determined. It would seem, however, that circulating haemocytes play a central role in both mechanisms through their involvement in immuno-recognition and in the processes of inactivation and elimination of foreign particles.

Prawn haemocytes

Prawns, as with most crustaceans, have at least three recognised blood cell types based on morphology and staining characteristics (Hearing & Vernick 1967; Hose *et al.* 1990; Jussila *et al.* 1998): granulocyte (also known as large granule haemocyte or eosinophil); hyalinocyte; and semi-granulocyte (small granule haemocyte). Both semi-granulocytes and granulocytes adhere readily to glass and plastic and emit long filopodia, assuming a stellate shape as described by Newman & Feng (1982), Goldberg *et al.* (1984, 1986) and Barracco & Amirante (1992). Filopodia are apparently associated with surface adherence and the density and type of cytoplasmic granules in the haemocyte (Goldberg *et al.* 1986).

It has been generally accepted, but not proved, that the three morphological haemocyte types represent different developmental stages of one cell line, with the granulocyte being the terminal stage (Bodammer 1978; Mix & Sparks 1980; Jussila *et al.* 1998). Hose & Martin (1989) found that hyalinocytes initiate coagulation and lyse in the presence of bacterial toxins and seawater, while granulocytes and semi-granulocytes are involved with phagocytosis and encapsulation. By contrast, Van de Braak *et al.* (2002b) present evidence for two cell lines in prawns. Both cell lines differentiate into large and small granulocytes, with “hyalinocytes” being the immature or intermediate stage in both cell lines. Old and apoptotic haemocytes are believed to accumulate and aggregate in haemal sinuses of the lymphoid organ (Anggraeni & Owens 1998, reported in Hasson *et al.* 1999)

The location of the haematopoietic tissue is known (Bell & Lightner 1988) though its function is not well understood (but see Van de Braak *et al.* 2002b). In *Nephrops norvegicus* it shows a marked seasonal cycle of activity (Field & Appleton 1995). It is likely that this activity is

also mediated by the presence of pathogens, since haemocytes are not observed to divide once in circulation. It would seem that the ultrastructure, cytochemistry and activity of haematopoietic tissue in prawns is a neglected field.

Cellular defence mechanisms - foreign agents

Inflammation has been studied in considerable detail in penaeid shrimp (Martin *et al.* 2000). Injection of shrimp with carmine (a neutral contaminant) is followed by accumulation of carmine in the dorsal abdominal artery, ventral abdominal vein, heart and gills. By 30 h post-injection, carmine is only visible in the gills, heart and injection site (Fontaine & Lightner 1974). Histologically, the carmine forms tightly packed extracellular masses, at the injection site, which are infiltrated and phagocytised by haemocytes. Circulating carmine particles are then trapped by fixed phagocytes lining the blood vessels and in sinusoids of the gill filaments. These particles finally accumulate in the distal gill filaments and heart. Brown melanised nodules consisting of necrotic haemocytes containing phagocytised carmine develop in the periopods and as cysts in the connective tissues of the gill cover by a process of filtration rather than through the action of fixed phagocytes and are subsequently shed at moulting (Martin *et al.* 2000). Carmine containing haemocytes also migrate through the midgut epithelium and into the lumen of the antennal gland. Smith & Ratcliff (1980a,b) studied clearance of foreign agents from gills of the crab *Carcinus maenas*. They found that there were two mechanisms in operation: aggregation of haemocytes into 12-25 µm diameter clumps of 5 to 50 haemocytes containing trapped bacteria; and the formation of elongate, diffuse networks of phagocytic haemocytes in the gill blood sinuses. More modern methods were employed by Van de Braak *et al.* (2002a,c) who used monoclonal antibody stains to detect bacteria and virus in haemocytes following challenge injections of *P. monodon* with live *Vibrio anguillarum*. Their results showed that bacteria were rapidly cleared from the haemolymph and that granular haemocytes were present in higher numbers in tissues with WSSV infected cells.

Aggregation of semi-granulocytes and granulocytes is accomplished by a combination of binding by pseudopodia and humoral factors. This occurs in response to foreign agents such as *Vibrio* sp. (Johnson 1976; Newman and Feng 1982) and is the precursor to encapsulation for foreign agents too large to phagocytise (Krol *et al.* 1989). Aggregation is often accompanied by extensive pre-mortem clotting of plasma and, in severe cases, the aggregation and plasma clotting can obstruct haemolymph leading to massive focal necrosis (Johnson 1976). Haemocyte aggregation (as opposed to activation and migration) and melanisation was observed for bacteria but not viruses (Van de Braak *et al.* 2002a).

Phagocytosis is a defence employed when the foreign agent is smaller than the haemocyte. For larger particles, a multicellular defence is required to encapsulate the agent. Encapsulation reactions by haemocyte preparations obtained from *Panulirus interruptus* were studied by Hose *et al.* (1990) who showed that the reaction involved the semi-granular haemocytes and fibrocytes. Haemocytes (granulocytes and semi-granulocytes) cluster around the foreign body, forming encapsulations many cell layers thick. The outer cells retain a more normal shape while inner cells become flattened. Diffuse melanisation occurs in the compact core and in the intracellular matrix forming a thick brown leathery capsule. Such capsules are not resorbed. Haemocytes also cluster around clots which are presumably resolved in time.

Degranulation is also a neglected area of study in prawns. Observation of histological material shows that mature granulocytes appear to aggregate near foreign agents and degranulate in the same way as molluscan haemocytes. This process was studied in quahog

(*Mercenaria mercenaria*) by Mohandas *et al.* (1985) who showed that bacteria stimulate haemocytes to extrude intact lysosomes into the haemolymph, a process referred to as degranulation. The resulting release of lysosomal hydrolases is assumed responsible for associated host and non-host tissue damage (Feng 1988, Hose & Martin 1989, Watanabe 1999) and may be one mechanism by which bactericidal activity is seen to rise in lobster haemolymph after inoculation of formalin-killed bacteria (see Sinderman (1971) for review). Recent work on degranulation of human eosinophils has suggested that the eosinophils do not discharge granules to the cell surface (exocytosis) but undergo lysis (Watanabe 1999),

Stress-related opportunistic infections result in an observed reduction in haemocyte numbers presumably by attrition (Stewart *et al.* 1967; Stewart & Rabin 1970; Newman & Feng 1982; Field & Appleton 1995; Jussila *et al.* 1998). Low haemocyte counts result in long clotting times (Sinderman 1971). It should, however, be noted that the same effect can occur through an increase in blood volume, and such changes are seldom measured. Changes in haemocyte counts with moulting may also be volume related (Tsing *et al.* 1989).

Is haemolymph sterile? There is ongoing debate. While many hold that the presence of bacteria in the haemolymph is indicative of septicaemia (Lightner 1977) and is a common result of stress (Lightner 1988), bacteria can be isolated from haemolymph of apparently healthy crustaceans. These include *Procambarus clarkii* (Scott & Thune 1986), *Homarus americanus* (Cornick & Stewart 1966, 1968), *Callinectes sapidus* (Colwell *et al.* 1975), and *Penaeus vannamei* (Gomez-Gil *et al.* 1998). However, bacterial infection following stress can occur rapidly during capture and transport (Johnson 1976; Messick & Kennedy 1990) making it extremely difficult to ensure that unstressed 'healthy' crustaceans have been sampled. In addition, some bacteria, such as *Aerococcus viridans* var. *homari* appear to be difficult for the host to kill and eliminate (Stewart & Rabin 1970).

Cellular defence mechanisms- toxic insults

Toxins come from three main sources - environmental contaminants; toxins associated with foreign invaders (Bowser *et al.* 1981); and toxins resulting from tissue damage and haemocyte degranulation. Reactions to toxins have been studied using injected irritant substances such as turpentine (Fontaine *et al.* 1975). The heart is the organ most affected by circulating turpentine in the haemolymph (Fontaine *et al.* 1975). An acute inflammatory reaction produces melanized haemocytic nodules in the heart followed by influx of haemocytes and fibrocytes. Scar tissue is also formed as numerous collagen like fibres replace myocardial fibres in which numerous melanized nodules are interspersed. The myocarditis reported by Wada *et al.* (1994) in *Panulirus japonicus* may represent the result of such a toxic insult.

Conclusions

Rapid advances are being made in the understanding of the humoral mechanisms of host defence in decapod crustaceans. However, the histopathology of cellular defence mechanisms, though first studied over 100 years ago, is still poorly studied. The influence of environmental stress, nutritional status and moult status of the host on defence responses have only recently drawn attention (Jussila *et al.* 1998; Hall & van Dam 1998). These are all areas of critical importance to animal husbandry and production in aquaculture and are difficult to interpret in the absence of a basic understanding of the cellular defence mechanisms. Much remains to be discovered.

CHAPTER 3: MATERIALS AND METHODS

Source and collection of prawns

Representative samples of prawns were collected from populations in defined geographical zones of Western Australia over a three-year period between 1998 and 2001 (Figure 3.1; Table 3.1). Prawns examined during this study were derived from wild caught and research hatchery sources and represented clinically normal animals, animals remaining after research trials were complete, or in some cases, diagnostic submissions of diseased hatchery sourced animals. Due to the varied source of the prawns, the number examined was variable (Table 3.1.). Hatchery sources animals were all from broodstock collected in WA waters but the precise collection data were usually unavailable.

Processing and fixation of samples

The preservation of prawn tissue for histology is difficult. Adequate fixation is essential for accurate interpretation of the histological section yet fixation is complicated by the extremely fast onset of autolysis in prawn tissues. As emphasized by Lightner (1996), specimens should be fixed immediately on removal of the prawns from water. The method recommended by Lightner is to inject the fixative into the carapace of the live prawn. However, because of occupational health and safety issues associated with working at sea on small boats with syringe needles, we successfully resorted to slicing live prawns longitudinally (para sagittal) and immediately dropping the prawn halves in the relevant fixative. Comparison of prawns fixed by both methods showed no detectable difference in fixation of tissues.

Several suitable fixatives can be used, depending on the tissue type and staining characteristics required.

The most widely used general-purpose fixative for prawns is Davidsons fixative, recommended by Lightner (1996). Unfortunately Davidsons fixative contains acetic acid, which denatures nucleic acids and is therefore not very suitable for molecular techniques unless the tissues can be quickly washed and stored in 70% alcohol. An alternative fixative, less destructive to DNA, is to use 10% formalin, neutral buffered (NBF) with either borax or with seawater.

Tissues required specifically for bioassay (see page 25), or molecular diagnostic techniques (page 20) were not fixed at all but were immediately preserved in either saturated EDTA-DMSO saline (SED) buffer (for bioassay) or frozen in liquid nitrogen. SED buffer is superior to alcohol as a preservative (R. Overstreet, pers. comm.).

Recipe for SED Buffer

Dissolve 95 g tetrasodium EDTA in 700 ml distilled water
Adjust pH to 7.5 with glacial acetic acid
Add NaCl to saturation (allow salt to dissolve as completely as possible, usually 100-200 g)
Add 200 mL DMSO, make up to 1 l with distilled water

SED buffer is relatively non-toxic, non-inflammable and stores indefinitely at room temperature. Since the buffer contains salt (NaCl) a white precipitate may form which does not affect the ability of the buffer to preserve tissue.

Histopathological examination

Fixed samples for histology were dehydrated, embedded in paraffin wax, sectioned at 3 microns and stained with haematoxylin and eosin using standard techniques. For samples P-99-518 and P-99-621, prawns were pooled at sea into polythene bags of fixative with labels corresponding to shots of the trawl. On subsequent cutting-in for histology, the wax blocks were identified by pool number, not individual prawns and there were generally (but not always) two slides per animal (Table 3.2). This made it difficult to assess prevalence of lesions. Subsequent samples were always identified to an individual animal.

Feulgen staining for DNA was attempted for sections containing virus-like inclusions, but was not successful if tissues had been stored for any length of time in Davidsons before embedding in wax.

Histological sections were examined for normality and changes of histopathological significance were recorded for each organ or tissue.

Electron microscopy

Tissues for electron microscopy were obtained in two ways. Initially, promising pieces of tissue were cut from wax histology blocks and were then de-waxed with xylene, hydrated through an alcohol series, post fixed in osmium tetroxide (1% in 0.1 M phosphate buffer for 1 hour) washed, dehydrated and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips CM10 transmission electron microscope (TEM). Results were generally disappointing, except for virus in occlusion bodies.

Subsequently, only formalin or Davidsons fixed tissues were used, with small pieces of tissue being post-fixed in 1 % osmium tetroxide, dehydrated and embedded in Epon resin before semi-thin sections were cut and stained with toluidine blue. Semi-thin sections were examined for areas of interest and only promising blocks were then ultra-thin sectioned, stained using uranyl acetate/lead citrate, and examined with a Philips CM10 TEM. This method was time and labour intensive, but resulted in acceptable pictures.

White Spot Syndrome Virus survey

Advice from the CCEAD was that the likely prevalence of white spot in an infected population of prawns or crabs would be far in excess of 15%. Therefore testing 30 prawns/crabs per "site" was recommended based on sampling at least 30 sites in the State. This sampling strategy was therefore adopted in Western Australia.

Molecular methods

Prawns were tested for the viruses WSSV, Yellow head (YHV), Gill-Associated Virus (GAV), Hepatopancreatic Parvo Virus (HPV) and Monodon Baculovirus (MBV) using various molecular methods (see Table 3.3)

For the detection of WSSV, a standard two-step nested PCR assay was used together with a Real-Time PCR, and/or a WS5 PCR for degraded DNA. The standard two-step nested PCR used is the OIE recognized method of Lo *et al.* (1996, 1997) and this amplified a 1st-step fragment of 1447 bp, and a nested fragment of 941 bp. This was used as the primary standard. The WS5 PCR developed by CSIRO Indooroopilly involved a self-nesting reaction yielding a reaction product of about 200 base pairs in a single amplification, and was included primarily for amplification from samples containing degraded DNA. It was used on all samples tested

by the two-step PCR, even if the DNA had not degraded, as confirmation of negative results obtained from the OIE recognized standard two-step nested PCR.

The integrity of the DNA extracted was analysed with a third PCR assay. This was a Decapod standard PCR that targets a 830 bp and 240 bp fragment of DNA from the 18S ribosomal RNA gene, developed by CSIRO Indooroopilly as a modification of the Decapod 18S assay recognized by the OIE.

If the DNA is degraded, there will be no 830 bp fragment amplified, indicating that the DNA may be too degraded to be amplified by the standard two-step nested PCR. In this case, the WS5 standard PCR can be used to amplify a self-nesting reaction product of approximately 200 bp from degraded DNA.

DNA extractions were performed using “DNAzol Reagent” (Gibco), as this was found to give slightly improved results compared to use of the buffers described in the OIE methodology¹. A negative control reaction was included with each test run, although a positive control reaction was included only occasionally in order to minimize potential contamination of the laboratory with previously amplified products

Although no laboratory standardization or comparison program was undertaken prior to the testing program, the Fish Health Laboratory did have access to a positive control, which consisted of a sample of imported frozen river prawns originating in Indonesia.

Any samples that tested positive at the Fish Health Laboratory in Western Australia were submitted to the Australian Animal Health Laboratories (AAHL, Geelong) for confirmatory testing.

¹ The method was subsequently developed into the Australian Standard Diagnostic test for WSSV, and is available on the Australian Government Department of Agriculture Forestry and Fisheries website.

TABLE 3.1. Source, identification, history and number of prawns examined in this study. (NBF = neutral buffered formalin; Dav = Davidsons fixative; SED = SED buffer; DNAzol = DNA extracted with commercial reagent; WSSV, GAV, YHV are viruses)

Species	Origin	History	No.	Fixative	Date of collection	Lab. No.	Notes
<i>M. latisulcatus</i>	Exmouth Gulf	trawled		Frozen -80; Dav	Oct 1998	P99-518	Many samples lost
<i>P. esculentus</i>	Exmouth Gulf	trawled		Frozen -80; Dav	Oct 1998	P99-518	Many samples lost
<i>P. esculentus</i>	Shark Bay	trawled		Dav	Nov 1998	P99-621	
<i>M. latisulcatus</i>	Shark Bay	trawled		Dav	Nov 1998	P99-621	
<i>P. esculentus</i>	Exmouth Gulf	trawled	36	Dav; SED	Nov 1999	P99-4005	
<i>M. endeavouri</i>	Exmouth Gulf	trawled	403	Dav; SED	Nov 1999	P99-4005	
<i>P. esculentus</i>	Shark Bay	trawled	30	SED	27 July 2000	FH00-119	Replace lost prawns, also lost
<i>M. latisulcatus</i>	Shark Bay	trawled	31	SED		FH00-119	Replace lost prawns, also lost
<i>P. monodon</i>	Broome	captive broodstock	44	12 in Dav; 5 NBF; remainder frozen	Sept 2000	P-00-2748	28 tested for WSSV; 15 tested fro GAV
<i>P. esculentus</i>	Exmouth	research	2		Oct. 2000	AS-00-3695	
<i>P. esculentus</i>	Exmouth	research	150		Nov 2000	AS-00-4406	Post-larvae
<i>P. esculentus</i>	Exmouth	research	150		Nov 2000	AS-00-4407	
<i>P. monodon</i>	Camarvon	research	3	?	Sep 2001	AS-01-2971	All -ve for GAV
<i>P. monodon</i>	Broome	captive broodstock	23	11 for PCR	Sep 2001	AS-01-2971	WSSV,GAV,

Species	Origin	History	No.	Fixative	Date of collection	Lab. No.	Notes
<i>P. merguensis</i>	Wyndham	research	100	Dav	Feb 2001	FH01-45	Also WSSV and YHV testing
<i>M. latisulcatus</i>	Wyndham	research	30	DNAzol	Jan 2001	FH01-44	-ve for YHV, WSSV
<i>P. merguensis</i>	De Grey	trawled	190	Dav	May 2001	AS01-1535, 1540-42	
<i>P. monodon</i>	De Grey	trawled	22	Dav	May 2001	AS01-1535, 1540-42	
<i>M. latisulcatus</i>	Nickol Bay	trawled	30	Dav	May 2001	AS01-1535, 1540-42	
<i>P. esculentus</i>	Onslow	trawled	44	Dav	May 2001	AS01-1535, 1540-42	
<i>Metapenaeopsis sp.</i>	Eaglehawk Isl, Dampier	trawled	30	Dav	May 2001	AS01-1535, 1540-42	
<i>P. monodon</i>	Joseph Bonaparte Gulf	trawled	22	Dav	Nov 2001	AS01-4005	WSSV YHV
<i>P. monodon</i>	Broome hatchery post larvae	hatchery	8	Dav	Nov 2001	AS01-4006	FH01-270 diagnostic
<i>P. esculentus</i>	Exmouth hatchery post larvae	hatchery	11	Dav	Dec 2001	AS01-4232	FH-01-286 detailed result
<i>P. monodon</i>	Broome hatchery broodstock	trawled/ hatchery	37	Dav	Mar-2002	AS02-832	

TABLE 3.2. Numbers of prawns in each pool and number of slides cut from each pool for the *M. latisulcatus* and *P. esculentus* collected in samples P99-518 and P-99-621.

Pool	P99- 518				P99- 621			
	<i>M. latisulcatus</i>		<i>P. esculentus</i>		<i>M. latisulcatus</i>		<i>P. esculentus</i>	
	prawns	slides	prawns	slides	prawns	slides	prawns	slides
1	24	42	21	38	2	4	3	5
2	29	36	15	28	6	9	10	14
3	225	40	24	39	5	9	10	18
4	27	41	20	32	5	8	4	6
5	18	30	13	20	5	8	4	8
6	8	17	19	30	3	4	10	16
7	4	8	14	26	3	5	10	14
8	23	37	18	27	21	36	2	3
9	25	42	7	11	24	38	8	14
10	21	32	5	9	15	19	3	5
11	8	17	10	15	5	9	5	8
12					44	55	3	5
13					13	17	32	47
14					24	40	30	43
15					27	38	27	36
16					21	30	30	49
17					5	10		
18					5	12		
19					5	9		
20					5	7		
21					3	4		
22					5	7		
23					5	11		
24					5	6		
25					5	10		

TABLE 3.3. Molecular tests performed to detect certain viruses known to occur in prawns (ticks do not indicate occurrence in WA).

Virus	TEST PERFORMED			
	Standard PCR	Real-Time PCR	ISH	Dot Blot Hybridisation
WSSV	√	√		
YHV		√		
GAV	√			
HPV			√	
MBV				√

Late in 2001, the opportunity was taken to develop real-time PCR capability for WSSV, using the expertise and equipment available through the State Agriculture and Biotechnology Centre at Murdoch University. For this purpose, two additional PCR assays were developed according to the specific requirements of the real-time PCR technology. The first reaction used primers selected from the sequence of the 941 base pair fragment amplified by the OIE assay, which amplified a sub-fragment of about 150 base pairs. The second reaction used primers selected from an alternative sequence, and amplified a fragment of about 150 base pairs.

Prawns were tested for YHV using Real-Time PCR, using the method developed by Dr Surendran Selladurai (Saturn Biotechnology) in consultation with Department of Fisheries staff. A positive control (virus RNA) was available.

Prawns were tested for GAV using a standard RT-nested PCR that has been developed by CSIRO Indooroopilly and described by Cowley *et al.* (2000a). A positive control (Plasmid 618) was available.

A DiagXotics kit for detecting the presence of MBV by Dot Blot Hybridization method was used to confirm the presence or absence of MBV in tissue sections seen to have MBV-like inclusion bodies by light microscopy. The method used was the recommended protocol accompanying the kit, with positive controls also provided with the kit.

Some samples were also tested, using a commercial DiagXotics kit to test for HPV by in-situ Hybridization (ISH), to confirm the presence or absence of HPV in some of the tissue sections seen to have HPV-like inclusion bodies by light microscopy. The method used was the recommended protocol accompanying the kit, with positive controls also provided with the kit.

Bioassay

Samples of prawn tissues were preserved for storage and transportation using either SED buffer or freezing in liquid nitrogen. These were then dispatched by international airfreight to Dr Robin Overstreet in California.

Data storage and analysis

Data collected in the study was collated and entered on Microsoft Excel spreadsheets. This database was used to determine the prevalence of parasites, pathogens and histopathological changes and to make comparisons between populations of prawns from different regions.



Figure 3.1. A. Map of Western Australia showing the places mentioned in the text. Port Hedland is just southwest of the De Grey River mouth.

CHAPTER 4: RESULTS OF SURVEY

PART I: THE DISEASE AGENTS

INTRODUCTION

A total of 1760 prawns were examined during this study, including 107 *P. monodon* (giant tiger prawn) from the north-west coast of Western Australia; 206 *F. merguensis* (banana prawns), 425 *M. endeavouri* (endeavour prawns), 246 *P. esculentus* (brown tiger prawns), and 242 *M. latisulcatus* (king prawns) all from the area Exmouth Gulf to Port Headland; 30 *Metapenaeopsis* spp. (coral prawns) from Dampier; and 191 *P. esculentus* and 302 *M. latisulcatus* from Shark Bay (Table 3.1).

The occurrence and nature of histopathological changes and microbial, protozoan and metazoan agents observed in the study from all species of prawns examined are discussed in the first part of this chapter. Information on the prevalence and distribution within tissues and between populations and regions for each species examined is presented in the second half of the chapter.

DISEASES CAUSED BY VIRUSES

White Spot Syndrome Virus survey

The results obtained from Western Australian samples are shown in Table 4.1 and are reported for six pools from each sample unless otherwise stated (tissue from 5 animals is combined into a pooled sample, so 30 animals are tested as 6 pools). Where the decapod 18S assay indicated that one or more pools of a given sample were of inadequate quality for testing, results are reported only for those pools that were of adequate quality.

All prawn samples obtained from Western Australia (which constituted about 70% of crustacean samples received, and included all commercially fished species as well as some incidental commercial species such as *Penaeus monodon*) were found to be negative for WSSV by both the OIE and WS5 assays. The reaction products were quite clean, with no evidence of non-specific amplification products.

The remaining 30% of samples tested were crabs of various sorts. The majority tested negative for WSSV with both assays, although the occurrence of reaction products was found to be a problem with regard to the interpretation of results (see Discussion, Page 68)

TABLE 4.1. Results of testing for WSSV in Western Australia (- = all 6 pools negative, or 0/6).

Lab. no.	Date collected	Sample	Species	Location	OIE method (Lo <i>et al.</i>)	WS5	Comments
00-190	23 Nov 00	“WA River Prawns”	<i>Metapenaeus spp.?</i>	Indonesia	+ (8/10)	+ (8/10)	Original frozen imports as supplied to Darwin
01-04	4 Jan 2001	30 prawns	<i>Metapenaeus spp.</i>	Exmouth	-	-	
01-05	4 Jan 2001	30 crabs	<i>Leptodius spp.</i> (xanthoid crabs)	Denham (Shark Bay)	+ (4/6)	-	Samples sent to AAHL (SAN 01-1921) Results: negative
01-06	4 Jan 2001	30 crabs	<i>Portunus pelagicus</i> (blue manna crabs)	Fishing Boat Harbour, Fremantle	+ (1/6)	+ (1/6)	
		20 crabs	<i>Portunus pelagicus</i>	Rous Heads, North Fremantle	-	+ (1/4)	
01-08	6 Jan 2001	30 crabs	<i>Metopograpsus spp.</i> (shore crabs)	Kailis processing plant, Exmouth	-	-	
01-14	10 Jan 2001	5 crabs	<i>Metopograpsus spp.</i>	Willy Creek, Broome	-	-	
01-15	10 Jan 2001	30 crabs	<i>Portunus pelagicus</i>	Albany	-	-	
01-17	10 Jan 2001	25 prawns	<i>Metapenaeus endeavouri</i> (endeavour prawns)	Carnarvon	-	-	
01-18	12 Jan 2001	30 crabs	<i>Leptograpsus variegates</i> (rock crabs)	Geraldton processing plant	-	-	

Lab. no.	Date collected	Sample	Species	Location	OIE method (Lo <i>et al.</i>)	WS5	Comments
01-26	18 Jan 2001	30 crabs	<i>Metopograpsus spp.</i>	Broome TAFE outlets.	-	-	
01-30	25 Jan 2001	30 crabs	<i>Metopograpsus spp.</i>	Willy Creek	-	-	
01-34	30 Jan 2001	11 crabs	<i>Metopograpsus spp.</i>	WAMRL, North Beach	-	-	
01-35	31 Jan 2001	10 crabs	<i>Metopograpsus spp.</i>	OceanWest Aquaculture, Exmouth	-	-	
01-42	27 Jan 2001	30 prawns	<i>Fenneropenaeus merguensis</i> (Banana prawns)	Wyndham	-	-	
01-44	8 Feb 2001	30 prawns	<i>Penaeus latisulcatus</i> (Western king prawns)	Wyndham (offshore)	-	-	
01-53	16 Feb 2001	30 prawns	<i>Penaeus latisulcatus</i>	Rous Head, Fremantle	-	-	
01-65	13 Mar 2001	30 prawns	<i>Penaeus monodon</i> (Black tiger prawns)	Broome	-	-	
01-102	30 Apr 2001	30 prawns	<i>M. latisulcatus</i>	Exmouth Gulf trawl 27 Apr.	-	-	Collected from different areas of Exmouth Gulf
		30 prawns	<i>P. monodon</i>	Exmouth Gulf trawl 29 Apr.	-	-	
01-110	4 May 2001	30 prawns	<i>Metapenaeus spp.</i>	North Cockburn Sound	-	-	
		30 prawns	<i>Metapenaeus spp.</i>	South Cockburn Sound	-	-	
		20 prawns	<i>Metapenaeus spp.</i>	Offshore from Cockburn Sound	-	-	
01-112	7 May 2001	30 prawns	<i>M. latisulcatus</i>	Mandurah Estuary	-	-	
01-124	17 May 2001	30 prawns	<i>Fenneropenaeus merguensis</i>	De Grey River, Port Headland	-	-	

Lab. no.	Date collected	Sample	Species	Location	OIE method (Lo <i>et al.</i>)	WS5	Comments
		30 prawns	<i>Metapenaeopsis spp.</i> (Coral prawns)	Eagle Hawk Island (near Dampier)	-	-	
		30 prawns	<i>P. monodon</i>	De Grey River, Port Headland	-	-	
		30 prawns	<i>M. latisulcatus</i>	Nickol Bay (near Dampier)	-	-	
		30 prawns	<i>P. esculentus</i>	Solitary Island, Onslow	-	-	
01-179	7 Aug 2001	30 crabs	<i>Leptodius sp.</i>	Cable Beach, Broome	n/a	n/a	Negative (real-time PCR)
01-181	8 Aug 2001	30 crabs	<i>Leptodius sp.</i>	Denham (Shark Bay)	-	-	Re-sampling of case 01-05.
01-190	17Aug2001	3 prawns	<i>P. monodon</i>	Carnarvon Hatchery	n/a	n/a	Negative (real-time PCR)
01-218	27 Sep2001	11 prawns	<i>P. monodon</i>	Broome hatchery (broodstock)	n/a	n/a	Negative (real-time PCR)
		9 prawns	<i>P. monodon</i>	Carnarvon hatchery (broodstock)	n/a	n/a	Negative (real-time PCR)

Two samples of crabs tested positive for WSSV by the OIE assay, one of which was also positive by the WS5 assay. The OIE assay yielded a reaction product that was consistent with the expected size of 941 base pairs from samples 01-05 (four pools out of six) and 01-06 from (one pool out of ten). DNA sequence analysis on the fragments excised from the gel confirmed that the sequence was that of WSSV or a WSSV-like virus in both cases. The WS5 assay gave negative results for all pools in sample 01-05, but positive results for sample 01-06 (two pools out of ten). Both samples were submitted to AAHL for confirmatory testing, which indicated negative results for WSSV for both samples overall, although there were some equivocal results with regard to specific pools.

Yellow Head Virus and Gill Associated Virus

All 60 *P. monodon* individually tested for GAV by 2 step nested GAV PCR were negative. Samples tested included 15 prawns from Broome (P-00-2748); 23 prawns from AS-01-2971 (Broome); 3 from Onslow and 19 from Joseph Bonaparte Gulf.

Monodon Baculovirus-like virus (MBV-like virus)

Species affected in Western Australia

Neither *P. monodon* nor the *Metapenaeopsis* spp. was infected with MBV-like virus. Prevalence in *M. endeavouri* was 72% and in *F. merguensis* was 6%. *P. esculentus* from Exmouth Gulf in October 1998 were infected but those sampled in May 2001 and from the De Grey River mouth (Port Headland) in May 2001 had no MBV-like virus. Post-larvae from a hatchery pilot run of *P. esculentus* at Exmouth were heavily infected in December 2001, suggesting that prevalence in wild stocks of *P. esculentus* from Exmouth Gulf may be either variable or seasonal but can be a problem in hatcheries. MBV-like virus was seen in both *M. latisulcatus* and *P. esculentus* from Shark Bay.

Clinical signs

In Western Australia MBV-like virus was associated with a mild mortality problem in juvenile *P. esculentus*, although losses were considered to be within normal operational limits. Serial sampling of affected tanks suggested that the juveniles were highly susceptible to infection, possibly causing mortality, up to about PL₁₀. Beyond PL₂₀ the juveniles appeared to lose their susceptibility to infection.

Histopathology

MBV-like virus forms large multiple spherical eosinophilic occlusion bodies within the hypertrophied nuclei of hepatopancreatic cells (Figure 4.1.A-D). The presentation of MBV-like virus in endeavour prawns was considered typical.

Electron microscopy

Occlusion bodies from an endeavour prawn (*M. endeavouri*) taken in Exmouth Gulf, November 1999, contain MBV-like baculovirus nucleocapsids measuring 205-286 x 29-35 nm (Figure 4.2, 4.3).

Molecular diagnostic techniques

Three animals with extensive MBV-like occlusion bodies were tested with the DiagXotics MBV Dot Blot Hybridization probe. No positive test samples were produced, though all

controls worked as expected. However, the MBV Dot Blot Hybridization kit was designed to be used for fresh tissue and may not have worked on the paraffin embedded tissue.

Hepatopancreatic Parvo-like Virus (HPV)

Species affected in Western Australia

No *P. monodon*, *M. endeavouri* or *Metapenaeopsis* spp. were infected with HPV. A total of 58 *F. merguensis* were infected with HPV, giving an overall prevalence of 28%. Of those affected, 50 were infected with HPV-like virus only, whilst 8 animals were also infected with MBV-like virus. *M. latisulcatus* from Exmouth Gulf were rarely infected (about 0.5%) as were about 5% of the *P. esculentus*. HPV was found in *P. esculentus* from Shark Bay but not in the *M. latisulcatus* from the same area.

Clinical signs

No clinical signs were observed.

Histopathology

HPV-like virus was observed mostly in the hepatopancreas, although also in the midgut, or both and were seen as prominent basophilic intranuclear inclusion bodies in hypertrophied nuclei of hepatopancreatic cells. Intranuclear inclusion bodies cause lateral displacement of the nucleolus and emargination of the chromatin. Note that those inclusions observed were usually basophilic, although there was variation in this respect, with some inclusions appearing more eosinophilic (Figure 4.4 A-D).

Electron microscopy

Though not well fixed, TEM showed typical HPV inclusion bodies with displaced nucleoli. The inclusion body consists of a disorganized mass of virus particles of about 22 nm diameter.

Molecular diagnostic techniques

The results of the DiagXotics HPV ISH are shown in Table 4.2, below. An HPV ISH positive section is shown in Figure 4.4.D.

TABLE 4.2. Results of testing various prawn samples for the presence of HPV with the DiagXotics kit. (LO = lymphoid organ, eni = eosinophilic virus like inclusions). Banana = *F. merguensis*; Endeavour = *M. endeavouri*)

Sample	Histology	HPV ISH	Comments
Banana AS-01-1539 #137A	Suspect strong HPV positive	++++	Very strong HPV ISH positive; used in subsequent tests as positive control (better than kit positive control)
Banana AS-01-1539 #43	Suspect mixed HPV and MBV in midgut and HP)	+	Few positives in HP
Banana AS-01-1539 #108	Suspect HPV positive	++	Some positive patches in HP
Banana AS-01-1539 #140A	Suspect HPV strong positive in midgut)	+++	Good HPV in midgut
Banana AS-01-1539 #65	Spheroids in LO, eni in midgut	0	No HPV detected
Banana AS-01-1539 #19A	Eni in midgut	0	No HPV detected
Endeavour P-99-4005 #125	Eni in midgut	0	No HPV detected*
Endeavour P-99-4005 #151ES	Eni in midgut	0	No HPV detected*
Endeavour P-99-4005 #158	Eni in midgut	0	No HPV detected*

*The sections P-99-4005 have been overfixed with Davidsons, and therefore the suitability of these tissues for ISH is questionable. The samples may not be negative for HPV – the overfixing of the samples may have restricted the probe in accessing the target DNA.

Large eosinophilic virus – like inclusions in midgut

Large eosinophilic virus-like inclusions were commonly observed in the midgut epithelium of all species except *M. latisulcatus* and *Metapenaeopsis* spp. They did not stain with the DiagXotics HPV ISH (Table 4.2) and, as *M. endeavouri* samples were apparently free of HPV in the hepatopancreas and yet regularly had these virus-like inclusions (Figure 4.5.) they were assumed, in the absence of evidence to the contrary, to be distinct from HPV. The precise cellular location (cytoplasmic or intranuclear) of these inclusions was difficult to determine from histology.

Species affected in Western Australia

Species affected in this survey included *M. endeavouri*, *F. merguensis*, *P. esculentus* and *M. latisulcatus*. Prawns from both Exmouth Gulf and Shark Bay were affected.

Clinical signs

No clinical signs were observed.

Histopathology

The eosinophilic virus-like inclusions were seen in hypertrophic midgut epithelial cells, often with a marginated basophilic body which was either the nucleus or nucleolus (Figure 4.5). Feulgen staining was attempted but the DNA had been denatured by the Davidsons fixative.

Electron microscopy

Not attempted.

DISEASES CAUSED BY RICKETTSIA, CHLAMYDIA OR MYCOPLASMS

No rickettsia, chlamydia or mycoplasmas were seen in this survey.

DISEASES CAUSED BY BACTERIA AND FUNGI

Bacterial disease was not a major feature of the samples examined, although minor lesions attributed to bacteria were observed in all species and in most sample groups. No fungi were seen in this survey.

Species affected in Western Australia

These included all species and all samples except one small sample of *Melicertus latisulcatus* from Dampier (AS-01-1540).

Clinical signs

Varies from no signs through a red appearance in moribund animals through to mortalities.

Bacteriology

The following species were isolated from moribund hatchery sourced PL's of *P. esculentus* in Western Australia: *Vibrio alginolyticus*, *Vibrio splendidus* and *Vibrio spp.*

Histopathology

Bacterial necrosis. Typical histological changes associated with bacteria in the hepatopancreas were observed in a small proportion of all prawn species from both wild-caught and hatchery sources. Affected digestive gland tubules have necrotic epithelial cells, surrounded by a moderate haemocyte inflammatory infiltrate. In advanced stages individual tubules form casts encapsulated with haemocytes and dense melanin deposits (Figure 4.6.A-E).

DISEASES CAUSED BY PROTOCTISTA

Unidentified intestinal flagellate

Two specimens of broodstock *P. monodon* received from the Broome Hatchery had enigmatic *Cryptobia*-like flagellates attached to the midgut (Figure 4.7.A)

Microsporidiosis

No microsporidians were found during the survey.

Haplosporidiosis

Species affected in Western Australia

Three cases were found during the survey, including two *M. endeavouri* from Exmouth Gulf in November 1999 (Figure 4.7.B, C.) and one *P. esculentus* from Exmouth Gulf in October 1998.

Clinical signs

No clinical signs were observed.

Histopathology

The haplosporidians are restricted to the hepatopancreas where multinucleate plasmodia occur in the epithelial cells of the tubules, replacing the cytoplasm with uninucleate stages. There is a haemocyte response to the infection involving melanization of the affected parts of the hepatopancreas. Mature spores were not observed.

One prawn from Exmouth Gulf in October 1998 had a multinucleate plasmodium stage lodged in a gill filament (Figure 4.7.B.).

Electron microscopy

The attempt to re-embed the histological material was unsuccessful. No recognisable structures were visible under the electron microscope

Gregarines

Species affected in Western Australia

Gregarines were commonly observed in the midgut of all species.

Clinical signs

No clinical signs were observed.

Histopathology

There were no pathological changes associated with their presence, however many gregarines in *P. esculentus* from Exmouth Gulf contained structures resembling MBV-like virus occlusion bodies (Figure 4.7.D.).

Electron microscopy

Not attempted.

MISCELLANEOUS METAZOAN PARASITES

Cestode plerocercoids

Species affected in Western Australia

All species were affected by tetrahyminid cestode plerocercoids. There were clearly several species of cestodes involved but no attempt was made to resolve the taxonomy.

Clinical signs

No clinical signs were observed.

Histopathology

Encysted trypanorhynch cestode larvae were commonly encountered, particularly associated with the ventral nerve chord (Figure 4.8.B, C.), but occasionally in other tissues. The cestode is encapsulated in a fibrous matrix of fibroblasts and collagen-like fibres oriented in parallel with the cyst wall.

Trematode metacercariae

Species affected in Western Australia

Trematode metacercariae were rare. Only two were recorded, both in *M. latisulcatus* from Exmouth Gulf (P-99-518) (Figure 4.8. D).

Clinical signs

No clinical signs were observed.

Histopathology

The metacercariae were observed encysted near the ventral nerve chord.

Nematode larvae

Species affected in Western Australia

Nematode larvae were recorded in *P. monodon*, *M. endeavouri* and *F. merguensis* from the northwest shelf and in *P. esculentus* and *M. latisulcatus* from Shark Bay (Figure 4.8. E, F). Nematode larval sections were consistent with the ascarid *Hysterothylacium* sp.

Clinical signs

No clinical signs were observed.

Histopathology

The larvae were observed occasionally encysted in the striated muscle, antennal gland and in the midgut wall, often with associated inflammation.

HISTOPATHOLOGY NOT DUE TO SPECIFIC PATHOGENS

Crystalline intranuclear inclusion bodies

Species affected in Western Australia

Two *P. esculentus* from Exmouth Gulf in 1998 had crystalline inclusions similar to those reported for *Baculovirus penaei*. However, in the absence of any confirmation of a viral aetiology by electron microscope or molecular diagnostic techniques, it is likely that the inclusions are not of viral origin. See Discussion on page 76.

Clinical signs

No clinical signs were observed.

Histopathology

The prawns' digestive glands show intranuclear tetrahedral inclusion bodies and nuclei show hypertrophy and chromatin margination (Figure 4.9.A, B).

Electron microscopy

Not attempted.

Molecular diagnostic techniques

Not attempted.

Crystalline deposits in Antennal gland

Species affected in Western Australia

One *M. latisulcatus* from Shark Bay in 1998 had crystalline deposits in the antennal gland.

Clinical signs

No clinical signs were observed.

Histopathology

The translucent crystalline shards were surrounded by a focal inflammatory response.

HISTOPATHOLOGY OF UNKNOWN AETIOLOGY

Focal inflammatory lesions

Haemocytic enteritis was observed in a *P. esculentus* from Shark Bay in 1999 (Figure 4.6.B.) which may have had a bacterial origin.

Focal haemocyte inflammatory infiltrates were observed in tissues of all prawns including nerve chord, lymphoid organ and testis (Figure 4.6.C, D). These were considered to have been due to bacterial infection, though no bacteria were seen in the lesions.

Small eosinophilic inclusion-like bodies

Species affected in Western Australia

P. monodon and *M. endeavouri*

Histopathology

These occurred in tegumental glands, gills and nerve chords and consisted of small faintly eosinophilic bodies associated with degeneration and apoptosis of cells. Affected cells often appeared to be sensory neurones associated with the cuticular epithelium (Figure 4.9.C, D).

Electron microscopy

Not attempted.

Lymphoid organ spheroids

Species affected in Western Australia

All species examined.

Clinical signs

No clinical signs were observed.

Histopathology

The lymphoid organs were commonly affected by spheroid formation, which ranged from mild to severe. Spheroids are well delineated spherical to irregularly shaped aggregations of hypertrophied cells with variable numbers of necrotic and vacuolated cells and cell debris (Figure 4.10.A-C.). See Discussion on page 68.

Electron microscopy

In some cases the spheroid cells contained small basophilic inclusions in the cytoplasm that was suggestive of a viral infection, however, TEM examination consistently failed to find virus particles within the inclusions. Their ultrastructure under TEM was suggestive of non-specific phagocytic lysosomes.

Ectopic spheroid syndrome

Species affected in Western Australia

All species examined.

Clinical signs

No clinical signs were observed.

Histopathology

Occasional ectopic spheroids were commonly observed in many individuals of all species (Figure 4.10. D, E). A syndrome of many ectopic spheroids in three or more tissues was recorded as “widespread” and considered as a specific syndrome. The tissues most commonly affected included cuticular glands lying just below the cuticular epithelium, connective tissues, nerve chord, gills and heart. Endeavour prawns were considerably less affected than other species.

Nerve chord syndrome

Species affected in Western Australia

This lesion was seen in the nerve chords of all species in both the northwest shelf and Shark Bay.

Histopathology

Nerve chords were universally affected by focal inflammation and neuronal loss, ranging from moderate to severe (Figure 4.11 A, B). Individual neurones were commonly observed degenerating and undergoing phagocytosis, which was followed by loss of the neurone and its replacement by infiltrating haemocytes, which eventually formed an ectopic spheroid.

Clinical signs

The severity of the pathological changes was sufficient to suggest that clinical consequences would have been expected in the live animals, however no such abnormalities were observed in the three sample groups of captive *P. monodon* broodstock from the Broome TAFE hatchery. It is therefore unlikely that the nerve chord lesion is a cause of clinical disease in *P. monodon*.

Stages of an unidentified metazoan parasite were commonly found in the nerve chords of all species, although not in all individuals. There was no suggestion of the parasites being causally associated with the nerve chord lesion in any species or sample group. Early stages of the same lesion were clearly evident in *P. esculentus* PL samples from a WA hatchery, prior to their move out of high health conditions in the hatchery. There was no evidence of the cause of the lesion in the PLs either, but they were free of metazoan parasites histologically.

BIOASSAYS

Unfortunately, the first shipment sent from Western Australia to Dr Overstreet was lost in transit. A second shipment of replacement samples, sent through a separate freight forwarding company was also lost in transit. After September 2001, freight forwarding to the USA became impractical and the bioassay component of the project was discontinued.

PART II: THE HOSTS

PENAEUS MONODON (GIANT TIGER PRAWNS)

A total of 117 animals were examined, including 20 caught from around Onslow in May 2001 and submitted directly (AS-01-1535), and 97 originally caught on the north-west shelf between Exmouth and Broome but kept as broodstock at Broome TAFE prior to submission. One batch of post-larvae (n=177) from a production run at Broome TAFE that suffered losses was examined and included.

No evidence of HPV or MBV was observed in these *P. monodon*, nor were there any eosinophilic virus-like inclusions in the midgut epithelium.

The distribution of various disease agents and abnormalities seen in the *P. monodon* samples screened can be seen in Table 4.3.

TABLE 4.3. Presence of various conditions noted in the adult *P. monodon* prawns screened in this study. (ELI= eosinophilic virus-like inclusions; Seci = small eosinophilic inclusion-like bodies; HP=hepatopancreas)

Sample No.	AS-00-2748	AS-01-1535	AS-01-2971	AS-01-4005	AS-02-832
ELI	0	15%(3/20)	4%(1/23)	0	0
Trypanorhynch cestodes	33% (12/36)	35% (7/20)	30% (7/23)	33% (5/15)	22% (5/23)
Nematode larvae	0	5% (1/20)	0	27% (4/15)	9% (2/23)
Bacterial necrosis in HP	22% (8/36)	10% (2/20)	48% (11/23)	13% (2/15)	0
Inflammatory lesions	50%(18/36)	15%(3/20)	13%(3/23)	40%(6/15)	13%(3/23)
Seci	0	0	0	27% (4/15)	43% (10/23)
Lymphoid organ spheroids	72% (26/36)	16% (3/19)	91% (21/23)	87% (13/15)	91% (10/11)
Ectopic spheroids	61% (22/36)	6% (1/18)	65% (15/23)	13% (2/15)	22% (5/23)
Nerve chord syndrome	62% (16/26)	79% (15/19)	100% (23/23)	73% (11/15)	100% (22/22)

The following disease agents were observed:

Eosinophilic virus-like inclusions

These were seen in the midgut in 4 animals (Table 4.3).

Unidentified intestinal flagellates

Two animals held in the Broome Hatchery as broodstock (AS-02-832) were found to have Cryptobia-like flagellates lining the midgut (Figure 4.7.A).

Bacterial disease

Histological changes associated with bacterial infection in the hepatopancreas were observed in a variable proportion of *P. monodon* from both wild-caught and hatchery sources (Table 4.3). Typical haemocytic nodules associated with bacterial infections were occasionally observed in other tissues, including nerve chord, lymphoid organ; and testis (Figure 4.6.A, C.)

Trypanorhynch cestode larvae

See Table 4.3. These were commonly observed in many tissues, particularly nerve chord, hepatopancreas, midgut, gills, muscle and connective tissues.

Nematode larvae

See Table 4.3. These were observed occasionally, encysted in the midgut wall and often with associated inflammation.

Focal inflammatory lesions

Mild acute and otherwise unexplained inflammation observed in various tissues, including nerve chord, muscle tissue, antennal gland, heart and cuticular epithelium.

Small eosinophilic inclusion bodies

These occurred in tegumental glands, gills and nerve chords (Figure 4.9.C). Four animals contained small eosinophilic inclusion-like bodies in tegumental glands. Two of the four animals also contained small eosinophilic inclusion-like bodies in the nerve chord. Apart from the inclusions themselves, the nerve chord lesion in affected prawns was not different from the nerve chord lesion affecting most animals of all species. All four affected animals also showed spheroids in the lymphoid organ, although so did most *P. monodon*. It was therefore not possible to associate small eosinophilic inclusion-like bodies with lymphoid organ spheroids or ectopic spheroids.

Lymphoid organ spheroid.

The majority of *P. monodon* samples examined showed spheroid formation in the lymphoid organ, which was scored 1 to 4 based on relative severity, although there was wide variation depending on geographical location and time of sampling (Table 4.4).

All broodstock samples received from the Broome TAFE (AS-00-2748; AS-01-2971; AS-01-4005; AS-02-832) had been collected in the same general area as AS-01-1535, but had been held in aquaria while spawning was attempted. They showed much more severe spheroid formation than the *P. monodon* submitted directly from the wild (Tables 4.3 and 4.4).

TABLE 4.4. Prevalence of lymphoid organ spheroids in the various *P.monodon* samples screened (4=severe).

	Relative score for lymphoid organ spheroids					% samples affected
	0	1	2	3	4	
AS-01-1535	16	3	0	0	0	16% (3/19)
AS-01-4005	2	6	5	2	0	87% (13/15)
AS-01-2971	2	2	8	5	6	91% (21/23)
AS-00-2748	10	0	12	13	1	72% (26/36)
AS-02-832	1	6	4	0	0	91%(10/11)
Prevalence %	30% (31/104)	16% (17/104)	28% (29/104)	20% (20/104)	7% (7/104)	70% (73/104)

Ectopic spheroid syndrome

Two sample groups of *P. monodon* (AS-00-2748 and AS-01-2971, Table 4.3) showed a high prevalence of this syndrome whilst the other two groups did not. Few of the broodstock from AS-01-4005 and the directly wild-caught animals of AS-01-1535 were affected. The tissues most commonly affected included tegumental glands lying just below the cuticular epithelium, connective tissues, nerve chord, gills and heart. The number of animals with widespread ectopic spheroids at each score value for lymphoid organ spheroids is shown in Table 4.5.

There was a significant relationship (Chi squared, $P=0.002$, $n=24$) between the relative abundance of spheroids in the lymphoid organ and the presence of ectopic spheroids in other tissues.

TABLE 4.5. Number of animals with ectopic spheroids in other tissues which also scored for lymphoid organ spheroids (1=rare, 4=most common).

	Relative	score	for	Lymphoid	spheroids
	0	1	2	3	4
ectopic spheroids present in other tissue	0	1	6	13	4
total no. with lymphoid spheroids	21	6	28	22	7

Nerve chord syndrome

All sample collections of *P. monodon* were highly affected by inflammation and neuronal loss in the nerve chords. Affected animals showed moderate to severe changes. Individual neurones were commonly observed degenerating and undergoing phagocytosis, followed by loss of the neurone and its replacement by infiltrating haemocytes (Figure 4.11). The severity of the pathological changes was sufficient to suggest that clinical consequences would have been expected in the live animals, however no such abnormalities were observed in the three sample groups of *P. monodon* broodstock that had been held at Broome TAFE. It is therefore unlikely that the nerve chord lesion is a cause of clinical disease in *P. monodon*.

***METAPENAEUS ENDEAVOURI* (ENDEAVOUR PRAWNS)**

A total of 425 animals were examined from Exmouth Gulf, all under the case number P-99-4005. No HPV was found in endeavour prawns (Table 4.6). The majority of endeavour prawns that were examined were found to be diseased with metazoan parasites in various tissues, mainly the nerve chord and hepatopancreatic epithelium, and/or MBV-like occlusion bodies in the hepatopancreatic epithelium. The significance of the presence of metazoan parasites is unknown as there was no pathology associated with their presence. However, due to the large number of endeavour prawns carrying metazoan parasites, the prevalence in various tissues and overall has been noted.

TABLE 4.6. Prevalence of various diseases or abnormalities seen in 425 endeavour prawns, sample number P-99-4005, from Exmouth Gulf. (Seci = small eosinophilic inclusion-like bodies)

Disease or condition	Prevalence(%) in Sample No. P-99-4005
MBV-like virus	72
HPV	0
Eosinophilic virus-like inclusions	12
Haplosporidian	0.5
Gregarines	10
Nematode larvae in midgut wall	8
Metazoan parasites	90
Seci	0.5
Lymphoid organ spheroids	0.5
Ectopic spheroid syndrome	7
Nerve chord syndrome	98

The following diseases were observed:

Monodon Baculovirus-like virus (MBV-like virus)

There were 305 affected animals out of 425, giving an overall prevalence of 72% in endeavour prawns from Exmouth Gulf. Presence of MBV-like virus was subjectively scored from 1 (low) to 4 (high). As evident from Table 4.7, only 29% of endeavour prawns that were screened did not show a presence of MBV-like occlusion bodies in the hepatopancreatic epithelium. Only 3% scored a high concentration of MBV-like occlusion bodies in the hepatopancreatic epithelium, and 42% of the samples tested showed a low concentration of MBV-like occlusion bodies in the hepatopancreatic epithelium.

TABLE 4.7. Presence of MBV-like occlusion bodies in the hepatopancreatic epithelium of endeavour prawns (4=highest).

	Relative score for MBV-like virus in hepatopancreatic epithelium				
	0	1	2	3	4
Presence in Hepatopancreas	122	179	60	52	12
Prevalence (%)	29	42	14	12	3

Eosinophilic Virus-like inclusions in midgut

These were commonly observed in endeavour prawn samples (Table 4.8). Out of a total of 425 midgut epithelium tissues screened, there were 59 that showed a presence of large eosinophilic inclusions, giving a total prevalence of 14%.

TABLE 4.8. Prevalence of eosinophilic inclusions (ENI) in the midgut epithelium of endeavour prawns. Crosses indicate severity of occurrence.

	No ENI	ENI +	ENI ++	ENI +++
Presence in midgut epithelium	366	53	2	4
Prevalence (%)	86	12.5	5	1

Haplosporidians

Two prawns had heavily infected hepatopancreatic tissue (Figure 4.7.B, C).

Gregarines

Of the 425 endeavour prawns examined, 42 (10%) had gregarines present in the midgut.

Metazoan parasites

90% of the endeavour prawns were affected by metazoan parasites (Table 4.9). There were 33 prawns (8%) with nematode larvae encysted in the midgut wall in histological section, often with associated inflammation.

TABLE 4.9. Prevalence of metazoan parasites in various tissues of endeavour prawns. For some of the endeavour prawns examined, more than one tissue was affected. (HP=Hepatopancreas)

	HP	Nerve chord	Muscle	Midgut	Connective Tissue	Antennal Gland	Gills
No. animals	220	331	8	17	5	2	19
Prevalence (%)	52	78	2	4	1	<1	4

Focal inflammatory lesions

These were not a major feature of endeavour prawn samples. Two animals showed inflammatory lesions in the heart.

Small eosinophilic inclusion-like bodies

These occurred in tegumental glands, gills and nerve chords. One animal showed changes in the tegumental glands that involved ectopic spheroid formation, and degeneration of cells that appeared to be sensory neurones associated with the cuticular epithelium. A couple of these cells had faintly visible eosinophilic structures in their cytoplasm that resembled the small eosinophilic inclusion-like bodies observed in tegumental glands and nerve chords of some *P. monodon* samples. No small eosinophilic inclusion-like bodies were found in the nerve chord or elsewhere of this prawn, and similar changes were not seen in any other endeavour prawns in this study.

Lymphoid organ spheroids

There were remarkably few of these in these endeavour prawn samples in comparison with other species. Only very mild spheroid formation was observed in 3 cases out of 425 animals examined.

Widespread ectopic spheroid syndrome

Very few endeavour prawns showed evidence of ectopic spheroid formation with only 6% showing signs of spheroid formation in the nerve chord, and only 1% showing spheroids in the cuticle (Table 4.10).

TABLE 4.10. Prevalence of widespread ectopic spheroid syndrome in endeavour prawns.

	Unaffected	Nerve chord affected	Cuticle affected
No. animals	394	26	5
Prevalence (%)	93	6	1

Nerve chord syndrome

The nerve chords of most endeavour prawns were affected by inflammation and neuronal loss, ranging from moderate to severe. Individual neurones were observed degenerating and

undergoing phagocytosis, which was followed by loss of the neurone and its replacement by infiltrating haemocytes, which eventually formed an ectopic spheroid.

Juvenile stages of an unidentified metazoan parasite were commonly found in the nerve chords of endeavour prawns (Figure 4.8. A), although not in all individuals. Parasites were not causally associated with the nerve chord lesion.

FENNEROPENAEUS MERGUIENSIS (BANANA PRAWNS)

A total of 206 animals were examined by histology from regions around Onslow, all under the case number AS-01-1539. The banana prawns tested as part of this study generally showed a low prevalence of MBV-like virus and a high prevalence of HPV. The significance of ectopic spheroids in numerous tissues is still unresolved. There was also a high prevalence of nerve chord syndrome (Table 4.11).

Table 4.11 Summary of the prevalence of abnormalities seen in banana prawns AS-01-1539.

Sample No.	AS-01-1539
MBV-like virus	6% (13/206)
HPV	28% (58/206)
MBV and HPV combined	4% (8/206)
Eosinophilic virus-like incl.	41% (83/203)
Metazoans	42% (86/206)
Lymphoid organ spheroids	35% (61/173)
Ectopic spheroid syndrome	7% (15/206)
Nerve chord syndrome	56% (116/206)

The following diseases were observed:

Monodon baculovirus-like virus (MBV-like virus)

There were 13 affected animals out of 206, giving an overall prevalence of 6%. Of those affected, 5 were infected with MBV-like virus only, and 8 had dual infections with HPV. Most occlusion bodies had the same typical appearance in banana prawns as in endeavour prawns, but less common non-typical forms were observed also.

Hepatopancreatic parvovirus (HPV)

A total of 58 banana prawns were infected with HPV-like virus, giving an overall prevalence of 28%. Of those affected, 8 animals were also infected with MBV-like virus. HPV was mostly observed in the hepatopancreas although was also common in the midgut, or both. Intranuclear inclusion bodies observed were usually basophilic, although there was variation in this respect, with some inclusions appearing more eosinophilic.

The diagnosis of HPV was confirmed by using a commercial HPV *in situ* hybridization kit, which targets HPV DNA in a histological section. HPV can be visualized by light microscopy as dark blue staining, easily seen in contrast with a light counterstain background, as shown by Figure 4.4.D.

Eosinophilic virus-like inclusions in midgut

These were commonly observed in banana prawn samples (41% of 203 animals). Many HPV-like virus inclusions were observed in midgut epithelium also, which were usually more eosinophilic than inclusions found in the hepatopancreas. The distinction was not always clear between the HPV-like virus inclusions observed commonly in these banana prawn samples and the eosinophilic virus-like inclusions observed commonly in banana prawns and most other species. However, some of the prawns showing these eosinophilic inclusions were tested for HPV using a commercial HPV ISH kit, and were non-reactive with the HPV probe.

Metazoan parasites

These were commonly observed encysted in many tissues, particularly nerve chord, hepatopancreas, midgut, gills, muscle and connective tissues. Juvenile stages of an unidentified metazoan parasite were commonly found in the nerve chords of these banana prawns. Nematode larvae were observed occasionally, often with associated inflammation.

Lymphoid organ spheroids

These were commonly observed in banana prawns, abundance was ranked from mild (1) to severe (4). Although viral infections were commonly observed in banana prawns, there was actually a negative association between lymphoid organ spheroids and virus in this sample (AS-01 1539; Table 4.12).

It is evident from Table 4.13 that many of the prawns with lymphoid organ spheroids were heavily infected, with only 2% having a mild grading of spheroids. Of the animals affected, 80% had a grading of at least 3, and only 20% had a grading of 2 or less, indicating that a severe case of lymphoid organ spheroids was considerably more common than a mild case of lymphoid organ spheroids.

Table 4.12. Sample AS-01-1539. Number of animals in each relative category rank for density of lymphoid organ spheroids (Los) together with numbers of those animals in each score which also had virus (MBV-like virus or HPV or both) or ectopic spheroids in tissues other than lymphoid organ.

Lymphoid organ spheroid (Los) score	Number of animals	Animals with concurrent virus	Animals with concurrent ectopic spheroids
0	40	19	4
1	3	1	2
2	7	1	2
3	36	7	26
4	13	2	9
TOTAL	99	30	43

Table 4.13. Prevalence of lymphoid organ spheroids in banana prawns AS-01-1539. A total of 173 animals were graded for the presence of spheroids in the lymphoid organ as mild (1) to severe (4), and those that had no spheroids were also recorded.

Presence of Lymphoid Organ Spheroids							
Grading:	1	2	3	4	None	No tissue found	Spheroids present (ungraded)
No. animals	4	8	36	13	112	30	61
Prevalence (%)	2	5	21	7	65	N/A	35

Ectopic spheroid syndrome

Affected about 5% of banana prawns, which was higher than other species. The tissues most commonly affected included tegumental glands lying just below the cuticular epithelium, but also the connective tissues, nerve chord, gills and heart. There was a correlation between the presence of lymphoid organ spheroids and ectopic spheroids (Table 4.14)

Table 4.14. Relative score for ectopic spheroids present in various tissues other than lymphoid organ.

	Relative score for ectopic spheroids				
Grading:	0	1	2	3	4
Present in other tissues	5	2	2	25	9
Total	59	4	8	38	13

Nerve chord syndrome

Most banana prawns were affected by inflammation and neuronal loss in the nerve chord, ranging from moderate to severe. Individual neurones were commonly observed degenerating and undergoing phagocytosis, followed by loss of the neurone and its replacement by infiltrating haemocytes and eventually forming an ectopic spheroid. The severity of the pathological changes was sufficient to suggest that clinical consequences would have been expected in the live animals, however it was not possible to determine in this study whether such was the case.

***PENAEUS ESCULENTUS* (BROWN TIGER PRAWNS)**

A total of 437 *Penaeus esculentus* prawns were examined including a sample of 246 from Exmouth Gulf (P-99-518; P-99-4005) and Onslow (AS-01-1541), and 191 from Shark Bay (P-99-621) (See Table 3.1). Four serial submissions were also received from a trial production run of *P. esculentus* post-larvae at a WA hatchery.

TABLE 4:15. Summary of the prevalence of abnormalities seen in *P. esculentus* during this survey. For samples from P-99-518 and P-99-621 pooling of samples during cutting in for histology (see Chapter 3) meant that accurate prevalence data are not available (Additional hatchery samples are excluded from this table).

Sample No.	P99-518	P99-621	P99-4005	AS01-1541
Number of animals	166	191	36	44
MBV-like virus	yes	yes	0	0
HPV	yes	yes	5	0
MBV and HPV combined	0	0	4	0
Eosinophilic virus-like incl.	yes	yes	28	77
Focal inflammation	yes	yes	22	18
Haplosporidian	yes	0	0	0
Gregarines	yes	0	6	0
Cestodes	yes	yes	92	77
Lymphoid organ spheroids	yes	yes	27	
Ectopic spheroid syndrome	yes	yes	44	20
Nerve chord syndrome	yes	yes	0	

Pathological changes observed in these samples consisted of the following:

Monodon baculovirus-like virus (MBV-like virus)

MBV-like occlusion bodies were not observed in any adult *P. esculentus* from P-99-4005 or from AS-01-1535. Infected animals were found in P-99-518 (Exmouth Gulf, in 2 of 275 slides, obtained from 166 animals) and P-99-621 (Shark Bay, in 6 of 291 slides obtained from 191 animals) (Table 4.15).

MBV-like virus was associated with a minor mortality problem in a pilot production run of juvenile *P. esculentus*, although losses (about 50%) were within normal hatchery limits and could not necessarily be attributed to the virus. Some batches showed severe infections with up to 100% prevalence whilst some batches were much less affected. Typical red, multiple, intranuclear occlusion bodies (Figure 4.1.B) were observed in affected animals that were histologically identical to the occlusion bodies observed in endeavour prawns. Serial examinations suggested that the juveniles were highly susceptible to infection, and possibly mortality, up to about PL10, but beyond PL20 the juveniles appeared to lose their susceptibility to infection.

Hepatopancreatic parvovirus (HPV)

HPV inclusions were found in the hepatopancreatic epithelium and/or midgut epithelium. Two *P. esculentus* out of 36 from P-99-4005 had clear HPV-like virus inclusions in the hepatopancreatic epithelium, which presented as typical large basophilic intranuclear inclusions. In P-99-518, 11 slides of 275 showed HPV while in P-99-621, 13 slides of 291 were infected.

Large eosinophilic virus-like inclusions

These were seen in the midgut epithelium and were commonly observed in these samples (Table 4.15). A few appeared to resemble HPV-like virus so closely they were tentatively described as such, although they were usually more eosinophilic than inclusions found in the hepatopancreas. It was not possible to determine definitively, from a histological study alone, whether all were representative of the same structures or even whether the cellular location was cytoplasmic or nuclear.

Haplosporidians

Three slides of *P. esculentus* were found with a haplosporidan infection (possibly one or two animals). One slide was of infected hepatopancreatic tissue while two had multinucleate stages lodged in gill tissues.

Gregarines

These were commonly observed in the midgut. There were no pathological changes associated with their presence, however many gregarines contained structures resembling MBV-like virus occlusion bodies (Figure 4.7.D.).

Metazoan parasites

Predominantly cestode trypanorhynch pleurocercae were commonly observed in many tissues, particularly nerve chord, hepatopancreas, midgut, gills, muscle and connective tissues. No pathology was associated with their presence. Nematode larvae were rarely found in the midgut wall of *P. esculentus* from Shark Bay (3 slides).

Lymphoid organ spheroids

These were commonly observed (Table 4.15). Samples from P-99-518 had 58 of 275 slides affected while P-99-621 had 86 of 291 slides affected.

Ectopic spheroid syndrome

These were relatively common in *P. esculentus* (Table 4.15). Samples P-99-518 had 68 slides of 275 affected, P-99-621 had 47 of 291 slides affected.

Nerve chord syndrome

The nerve chords of all sample collections of *P. esculentus* were universally affected by inflammation and neuronal loss, ranging from moderate to severe. Individual neurones were commonly observed degenerating and undergoing phagocytosis, which was followed by loss of the neurone and its replacement by infiltrating haemocytes, which eventually formed an ectopic spheroid. The severity of the pathological changes was sufficient to suggest that clinical consequences would have been expected in the live animals, however it was not possible to determine in this study whether such was the case.

Juvenile stages of an unidentified metazoan parasite were commonly found in the nerve chords of most species, and were particularly common in these samples. The nerve chord lesion was no less in animals that had no parasites however, so there was no suggestion of the parasites being causally associated. Early stages of the same lesion were clearly evident in PL samples also, prior to their move out of sterile conditions in the hatchery. There was no evidence of the cause of the lesion in the PLs either, but they were free of metazoan parasites histologically.

Crystalline intranuclear inclusion bodies

Two *P. esculentus* from Exmouth Gulf in 1998 had intranuclear tetrahedral crystalline inclusions in the digestive gland epithelial cells. Affected nuclei show hypertrophy and chromatin margination similar to those reported for *Baculovirus penaei* infection (Figure 4.9.A, B). However, in the absence of any confirmation of a viral aetiology by electron microscope or molecular diagnostic techniques, it is likely that the inclusions are not of viral origin. See Discussion on page 76.

***MELICERTUS LATISULCATUS* (KING PRAWNS)**

A total of 514 *M. latisulcatus* were collected from the Exmouth Gulf and 302 from Shark Bay. The king prawns tested as part of this study showed no MBV-like virus and only one prawn had HPV (in the hepatopancreas). Eosinophilic-like inclusions were not seen. One prawn from P99-621 had refractile crystals in the antennal gland.

Table 4:16. Summary of the prevalence of abnormalities seen in *M. latisulcatus* during this survey. For samples from P-99-518 and P-99-621 pooling of samples during cutting in for histology (see Chapter 3) meant that accurate prevalence data are not available.

Sample No.	P99-518	P99-621	AS01-1540
Number of animals	212	302	30
MBV-like virus	0	0	0
HPV	yes	0	0
Focal inflammation	yes	yes	0
Gregarines	yes	yes	0
Cestodes	yes	yes	26
Lymphoid organ spheroids	yes	yes	3
Ectopic spheroid syndrome	yes	yes	10
Nerve chord syndrome	0	yes	0

Focal inflammation

Focal inflammation, attributed to bacterial infection, was relatively common in P99-518 with 12 slides showing the condition, while in P99-612 only 2 slides were similarly affected. Two slides from P99-518 also showed focal myosytis. As animals were pooled (Table 3.2), slides do not necessarily represent individuals.

Metazoan parasites

Trypanorhynch pleurocercae were commonly encysted in the prawns, being found in 166 slides from P99-518 and 107 slides from P99-621. 26% of animals from AS-01-1540 were affected. In addition small spherical eggs with a thick chitinous wall were present in the gills, musculature, hepatopancreas and nerve tissues in 14 slides of prawns from P99-621.

Lymphoid organ spheroids

Were present in all samples in low numbers (96 slides from P99-518 and 77 slides from P99-621). In AS-01-1540 about 3% were affected.

Ectopic spheroid syndrome

Ectopic spheroids were seen in the gills on 9 slides from P99-518 and 24 slides from P99-621. and in 10% of the animals from AS-01-1540.

Nerve chord syndrome

Was very rare, with only 3 slides from P99-621 showing the condition.

Crystalline deposits in Antennal gland

One *M. latisulcatus* from Shark Bay in 1998 had crystalline deposits in the antennal gland. The translucent crystalline shards were surrounded by a focal inflammatory response.

***METAPENAEOPSIS* SPP. (CORAL PRAWNS)**

A total of 30 *Metapenaeopsis* spp. were examined, all from Eagle Hawk Island, Dampier (AS-01-1542). These prawns were heavily infected (25/30) with large numbers of encysted trypanorhynch metazoans, possibly of two types. They were free of MBV-like virus and HPV. Almost half of the sample (12/30) had focal inflammatory lesions in the hepatopancreas and gills and 5/30 had ectopic spheroids in the nerve chord or gills. Only two of the samples had lymphoid organs, and none had sections through the midgut. 3/30 were affected with the nerve chord syndrome seen in other species.

FIGURE 4.1

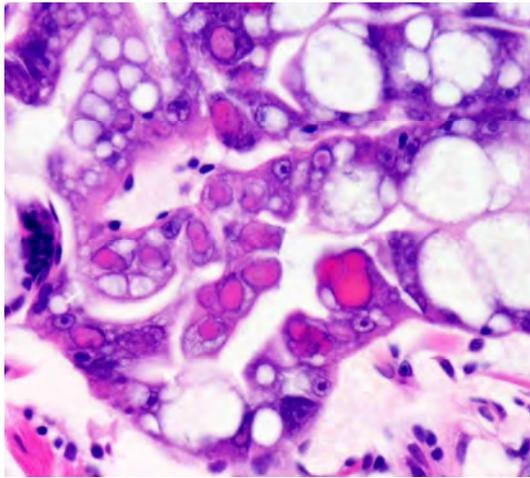


Figure 4.1. A: Spherical eosinophilic occlusion bodies of MBV in hepatopancreas of *M. endeavouri*, x 400.

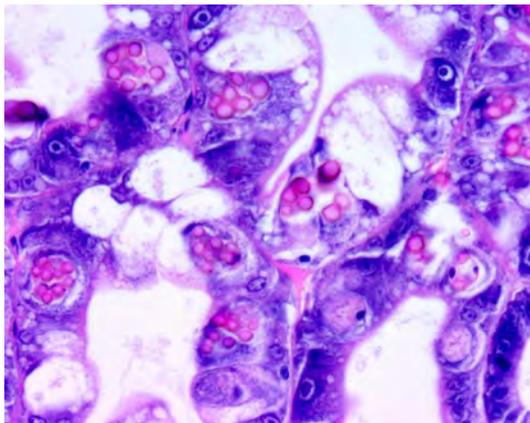


Figure 4.1. B: Spherical eosinophilic occlusion bodies of MBV in hepatopancreas of *P. esculentus* post-larvae, x 400.

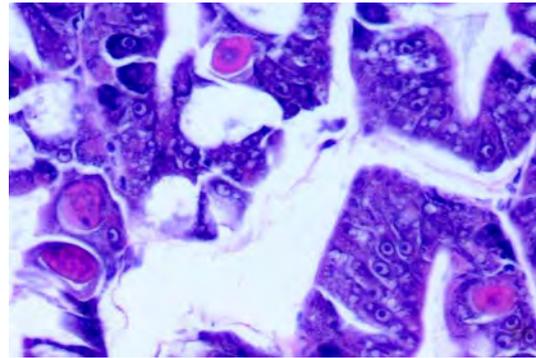


Figure 4.1. C: 'Smeared' atypical MBV seen in a small number of *M. endeavouri*. Compare appearance to Fig.4.1.B above, x 400.

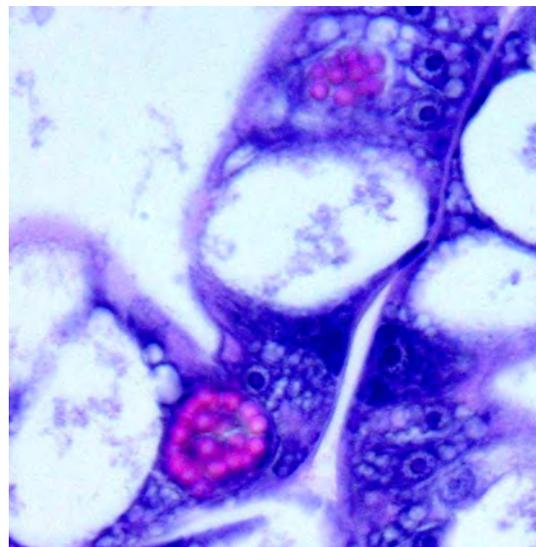


Figure 4.1. D: Atypical MBV in *M. endeavouri*, x 800.

FIGURE 4.2

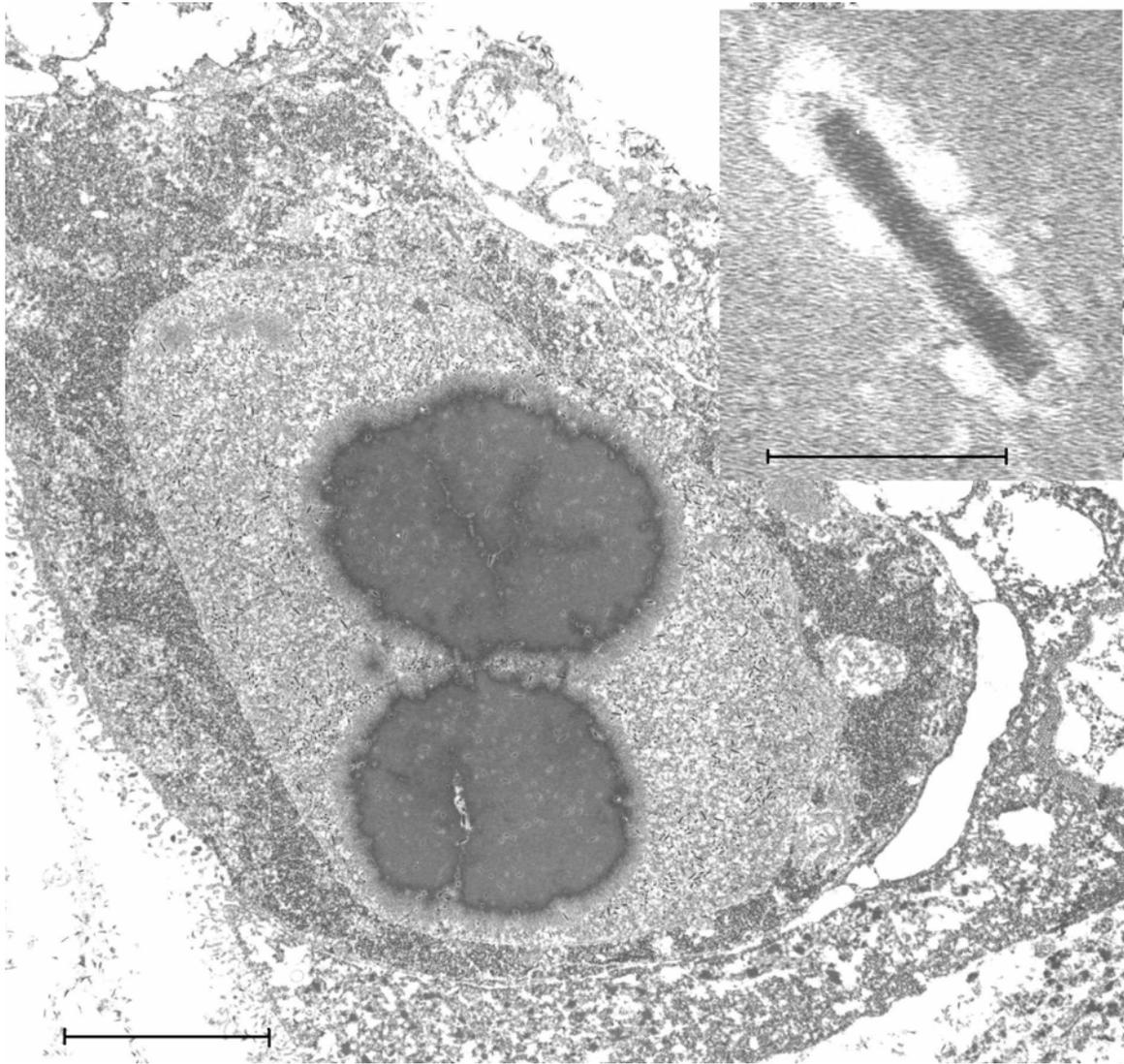


Figure 4.2. A. *M. endeavouri* hepatopancreatic cell with MBV occlusion bodies in nucleus. Viral particles (insert) can be seen throughout the cell. (Insert scale bar = 0.2 microns; scale bar = 4.4 microns) .

FIGURE 4.3

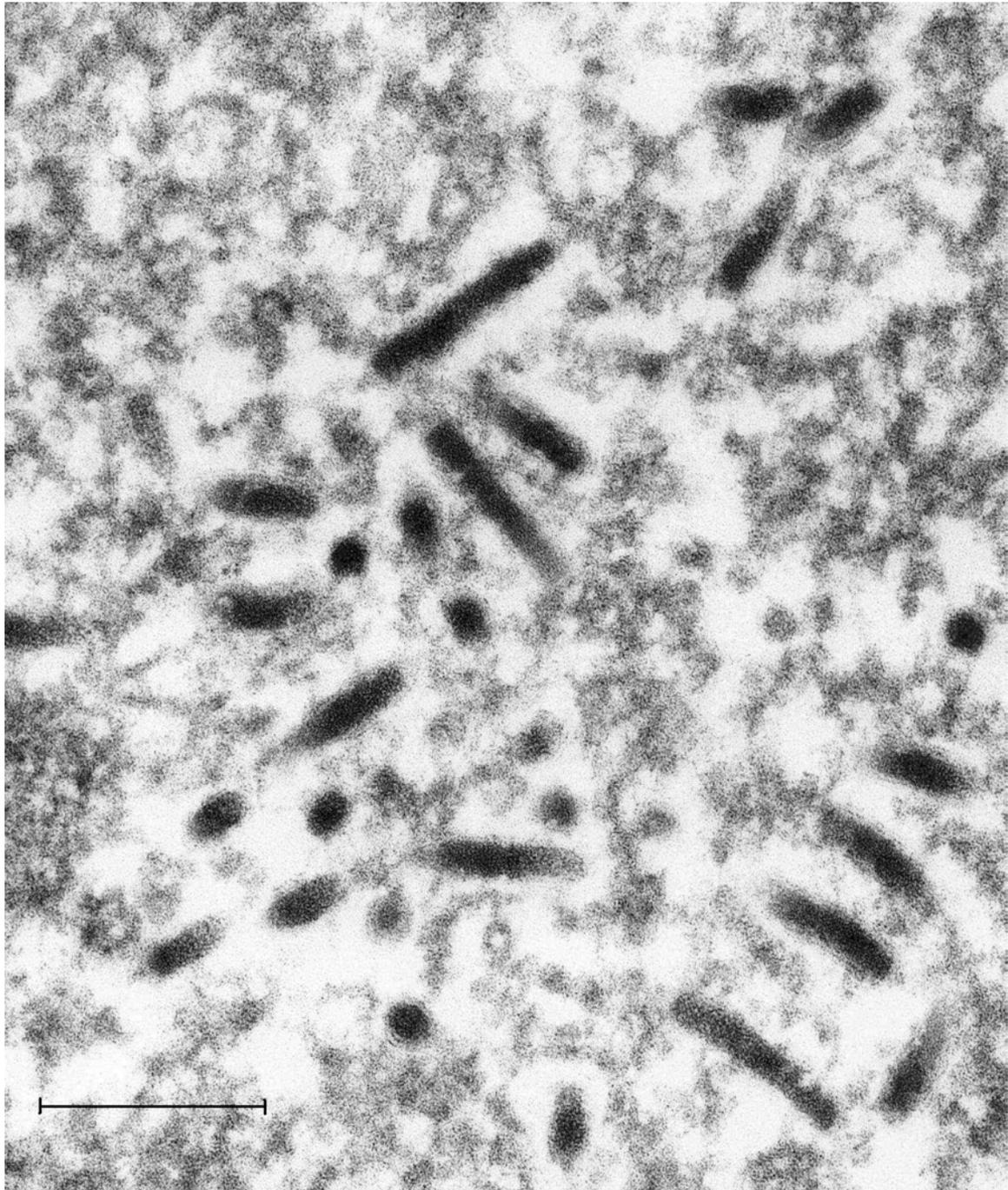


Figure 4.3. *M. endeavouri* MBV in cell cytoplasm, scale bar = 0.2 microns.

FIGURE 4.4

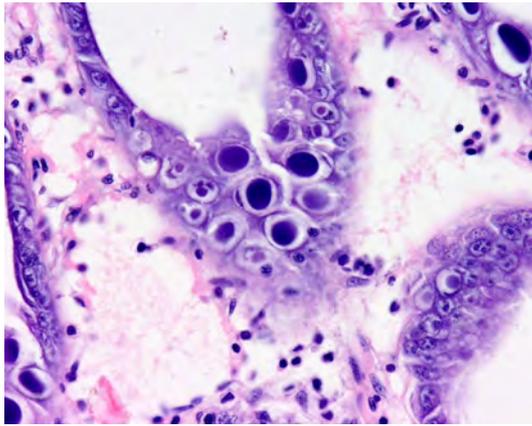


Figure 4.4. A: Basophilic inclusion bodies of HPV in hepatopancreas of *F. merguensis*, x 400.

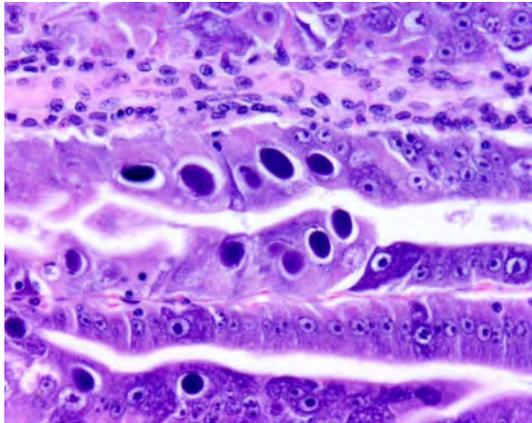


Figure 4.4. B: Basophilic inclusion bodies of HPV in *F. merguensis* midgut epithelium, x 400.

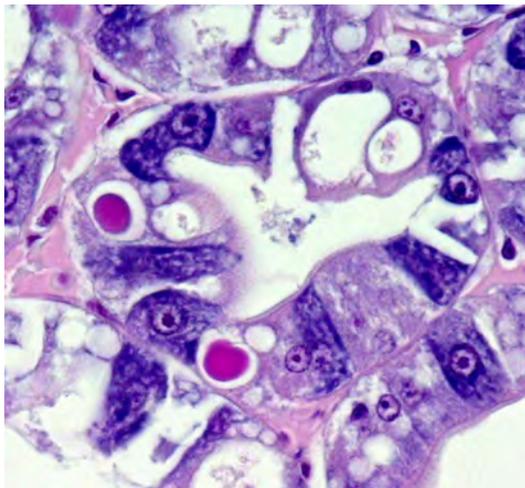


Figure 4.4. C: HPV inclusions showing variation in staining. *P. esculentus* hepatopancreas, x 400.

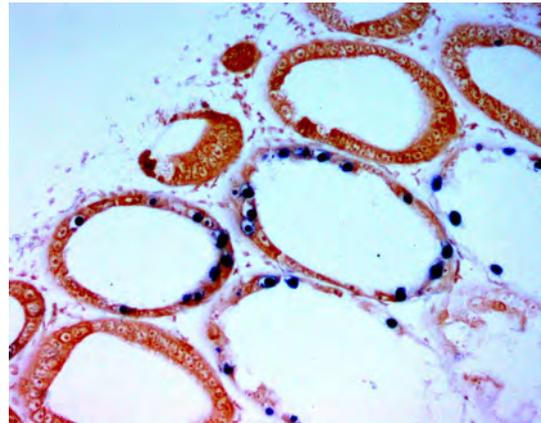


Figure 4.4. C : HPV ISH on *F. merguensis* viewed by light microscopy at x 200. The dark blue stained probe is confirmation that HPV DNA is present within the hepatopancreatic tissue. Counterstain is Bismark Brown.

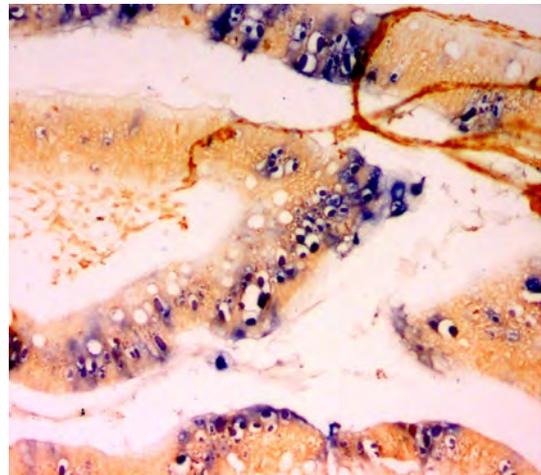


Figure 4.4. D: HPV ISH performed on *F. merguensis*. The virus is seen here in the midgut epithelium as dark blue HPV probe against a Bismark Brown counterstain, x 200.

FIGURE 4.5

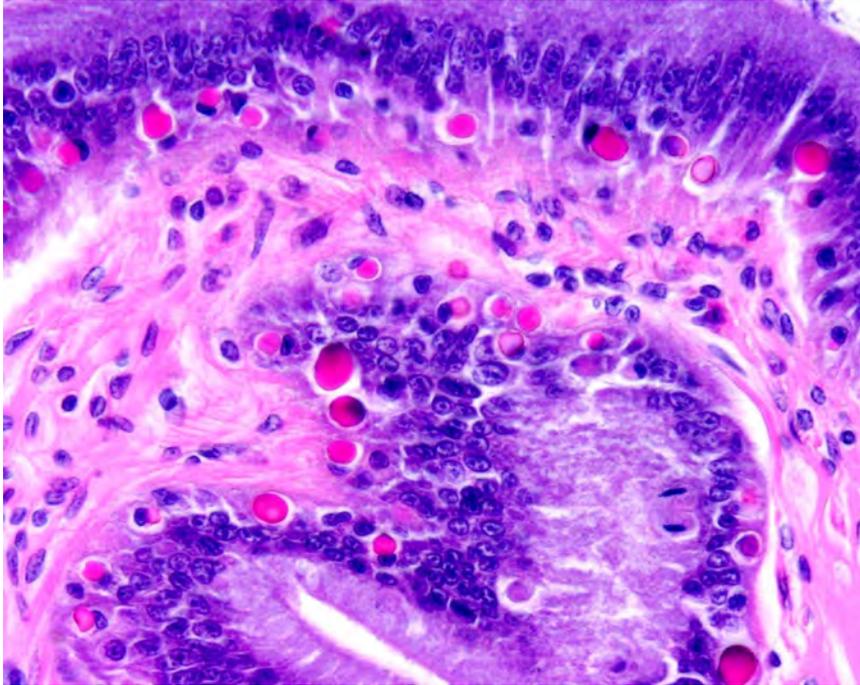


Figure 4.5. A: Eosinophilic inclusions in midgut epithelium of *P. esculentus*, x 400.

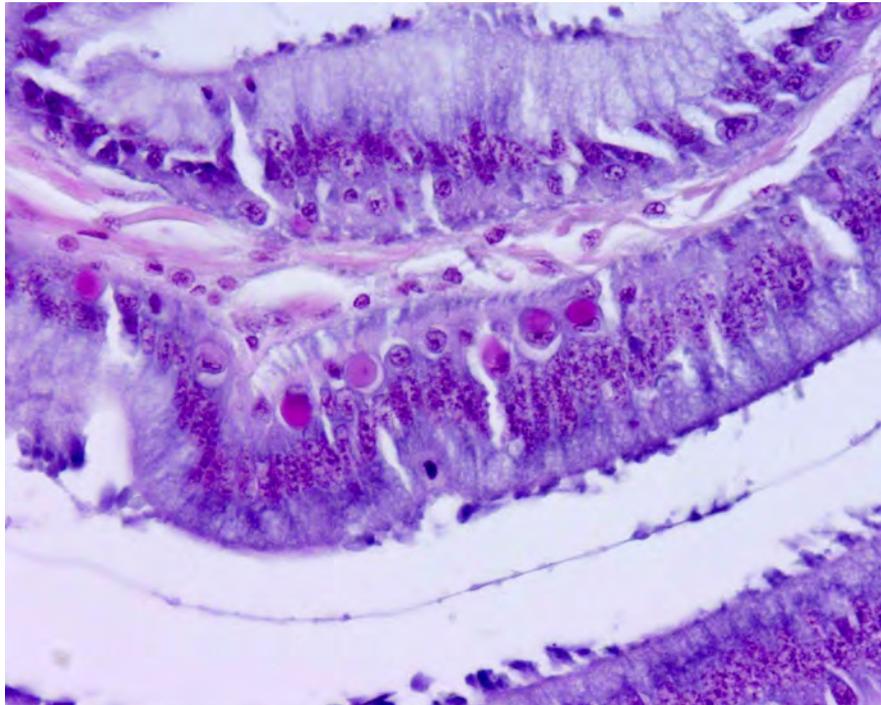


Figure 4.5. B: Eosinophilic inclusions in midgut epithelium, *P. esculentus*, x 400.

FIGURE 4.6

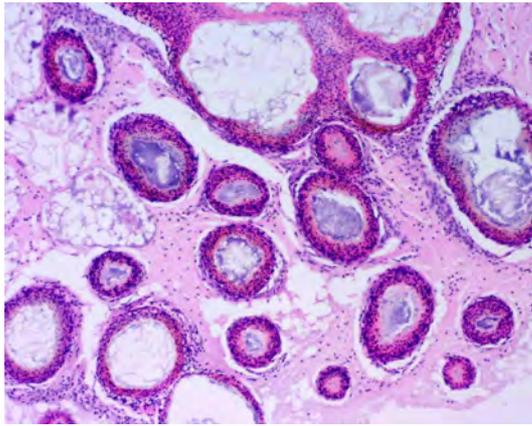


Figure 4.6. A: Casts of bacterial origin in hepatopancreas of *P. monodon*, x 100.

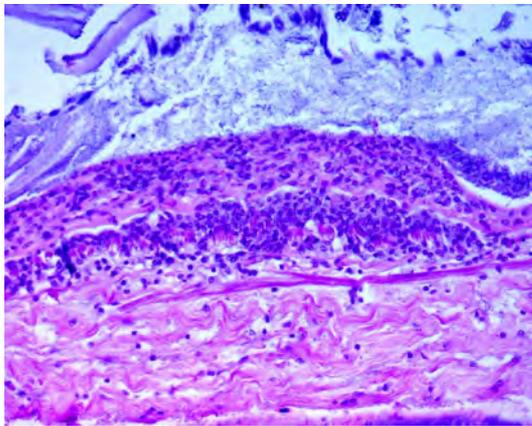


Figure 4.6. B: Haemocytic enteritis in *P. esculentus* from Shark Bay, x 400.

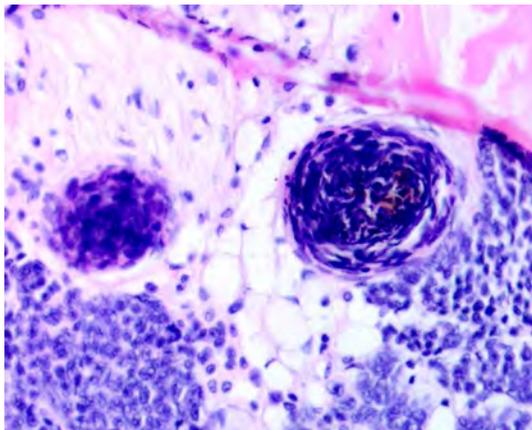


Figure 4.6. C: Bacterial nodules in testis of *P. monodon*, x 200.

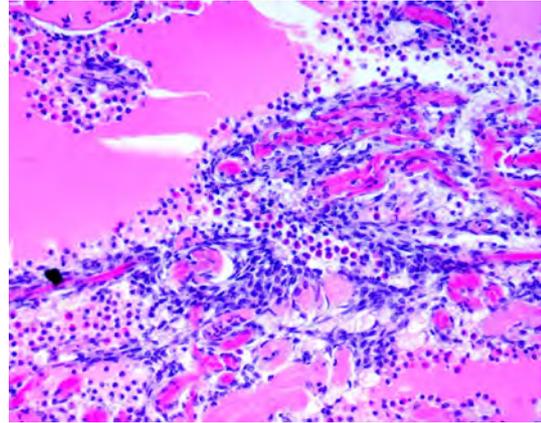


Figure 4.6. D: Heart lesion, *M. endeavouri*, Exmouth Gulf, suspect bacterial infection, x 200.

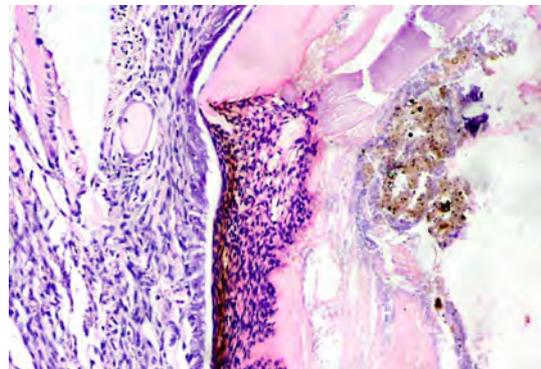


Figure 4.6. E: Inflammatory response following penetration of carapace by bacteria. *P. monodon* from Broome Hatchery, x 100.

FIGURE 4.7

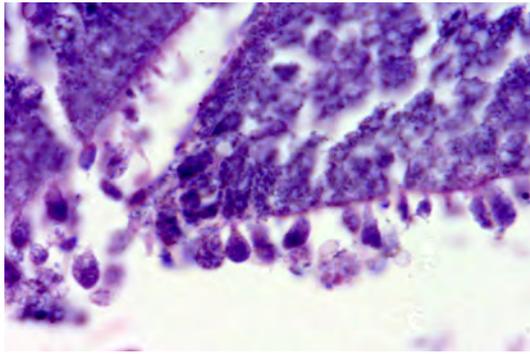


Figure 4.7.A. Protozoans attached to wall of midgut, *P. monodon*, Broome hatchery, x 1000.

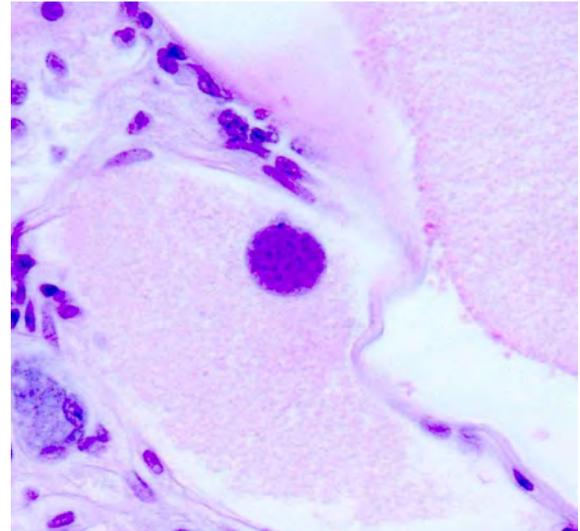


Figure 4.7. C: Haplosporidan multinucleate plasmodium stage from *M. endeavouri*, gill, Exmouth Gulf, x 400.

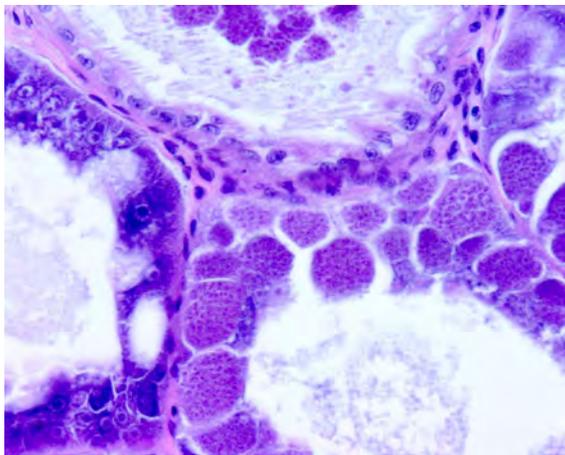


Figure 4.7. B: Haplosporidan from *M. endeavouri*, Exmouth gulf, x 400.

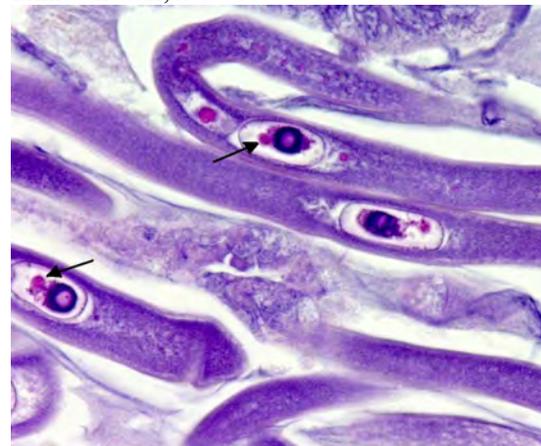


Figure 4.7. D. Gregarines in midgut of *P. esculentus*, with MBV-like bodies (arrows), x 400.

FIGURE 4.8

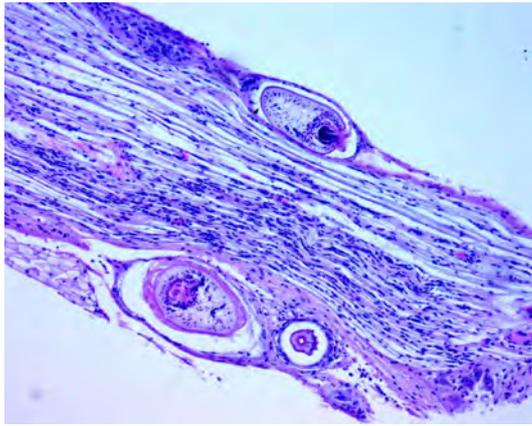


Figure 4.8. A: Metacystodes in nerve chord sheath of *M. endeavouri*, x 200.

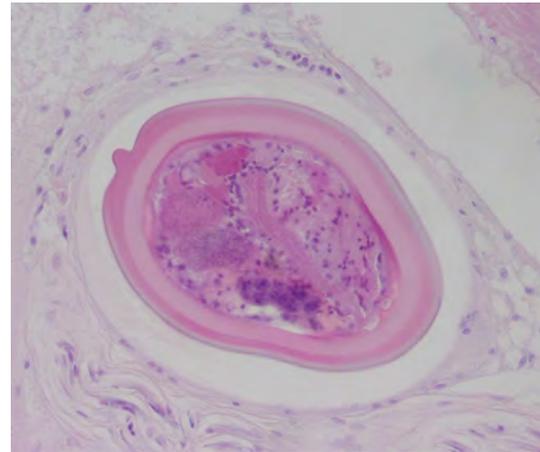


Figure 4.8. D: Trematode metacercaria encysted near nerve chord, *P. esculentus*, x 200.



Figure 4.8. B: metacystode in digestive gland, *P. esculentus*, Exmouth Gulf, x 100.

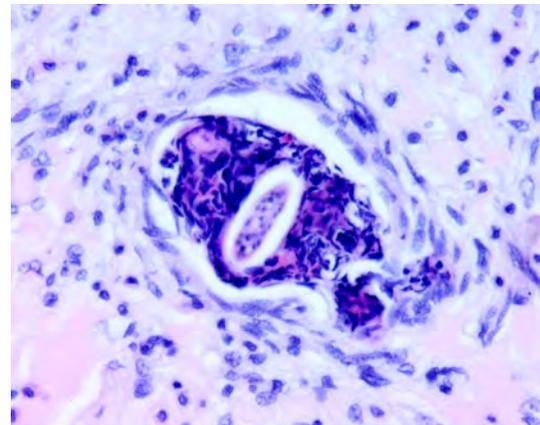


Figure 4.8. E: Nematode larvae encysted in midgut. *P. monodon*, x 400.

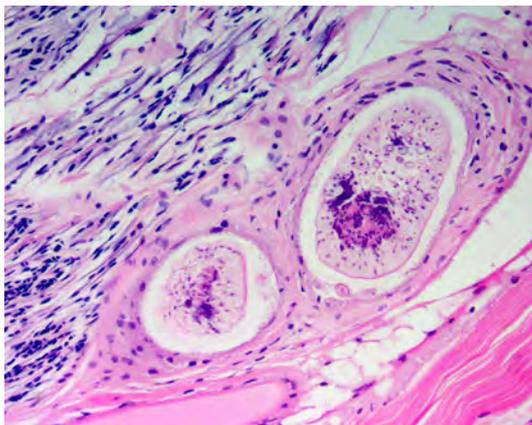


Figure 4.8. C: Metazoans adjacent to nerve chord, *F. merguensis*, x 200.

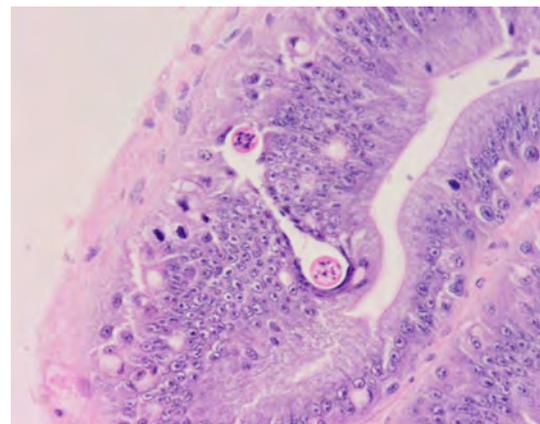


Figure 4.8. F: Nematode larvae (ascarid?) coiled in midgut of *F. merguensis*, x 200.

FIGURE 4.9

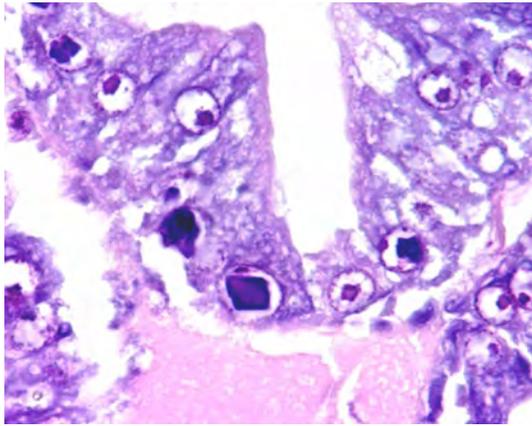


Figure 4.9. A: *P. esculentus* digestive gland crystalline inclusions, x 1000.

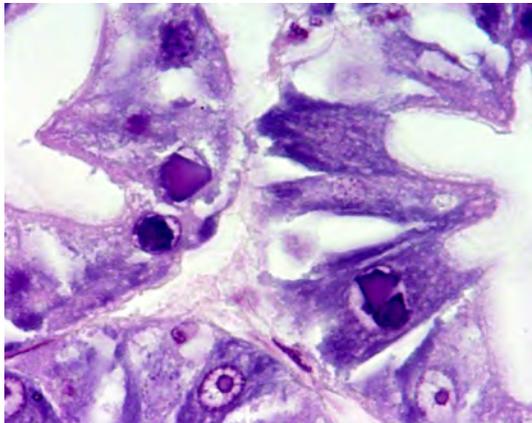


Figure 4.9. B: *P. esculentus* digestive gland crystalline inclusions, x 1000.

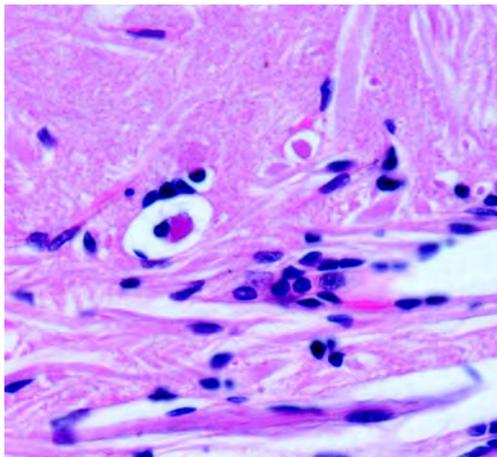


Figure 4.9. C: Small eosinophilic virus-like inclusion. Nerve chord, *P. monodon*, x 1000.

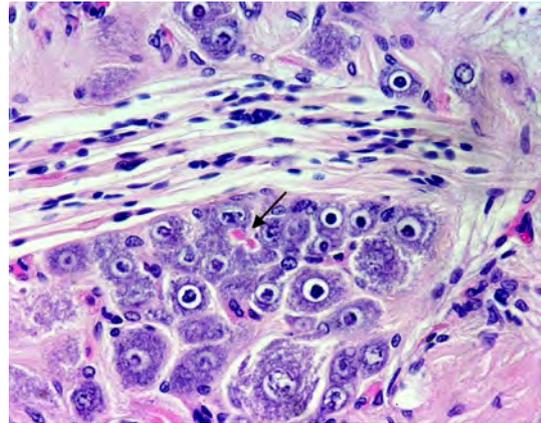


Figure 4.9. D. *P. esculentus* nerve with small eosinophilic bodies associated with apoptosis (arrow), x 400.

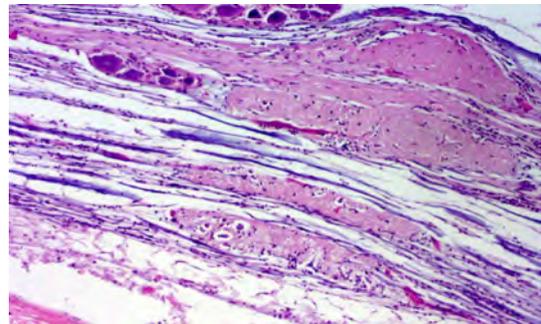


Figure 4.9.E. *P. esculentus* ex Exmouth Gulf, nerve lesion of unknown aetiology, x 100.

FIGURE 4.10

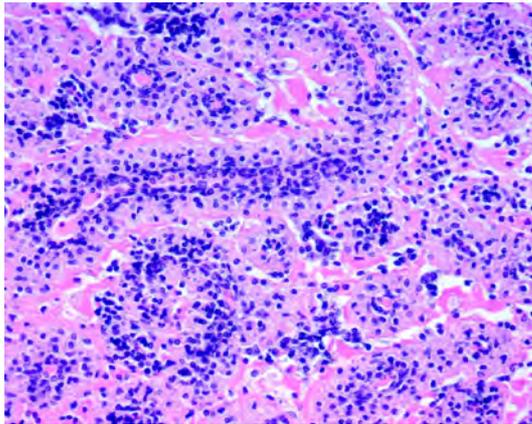


Figure 4.10. A: Mild lymphoid organ spheroids from *M. endeavouri*, Exmouth Gulf, x 200.

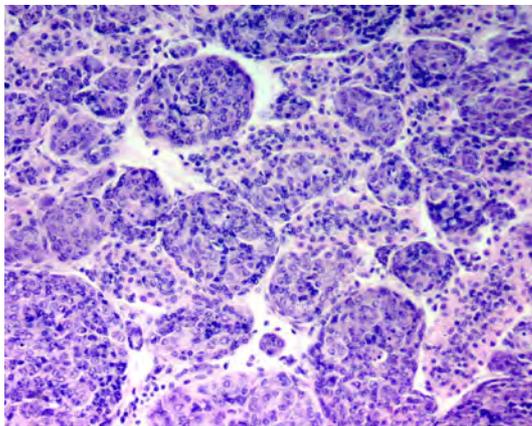


Figure 4.10. B: Lymphoid organ spheroids. *F. merguensis* infected with HPV, x 200.

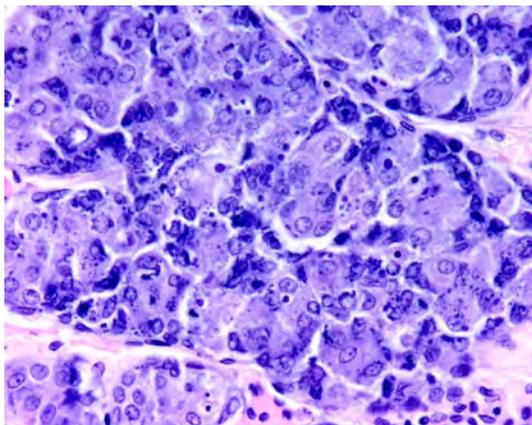


Figure 4.10. C: Severe case of lymphoid organ spheroids in *P.monodon*. Note basophilic inclusion-like bodies throughout the tissue, x 400.

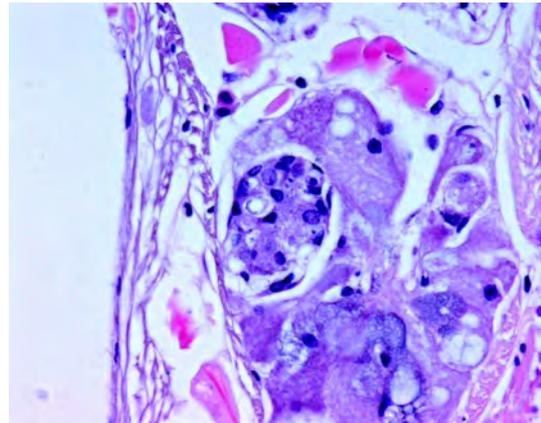


Figure 4.10. D: Ectopic spheroid in tegumental gland, *P. monodon*, x 400.

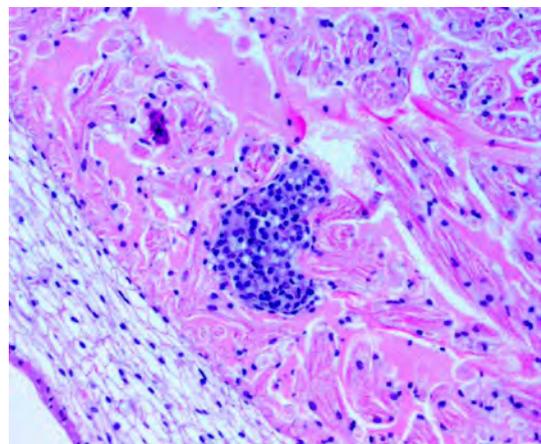


Figure 4.10. E: Ectopic spheroid in muscle, *P.monodon*, x 100.

FIGURE 4.11

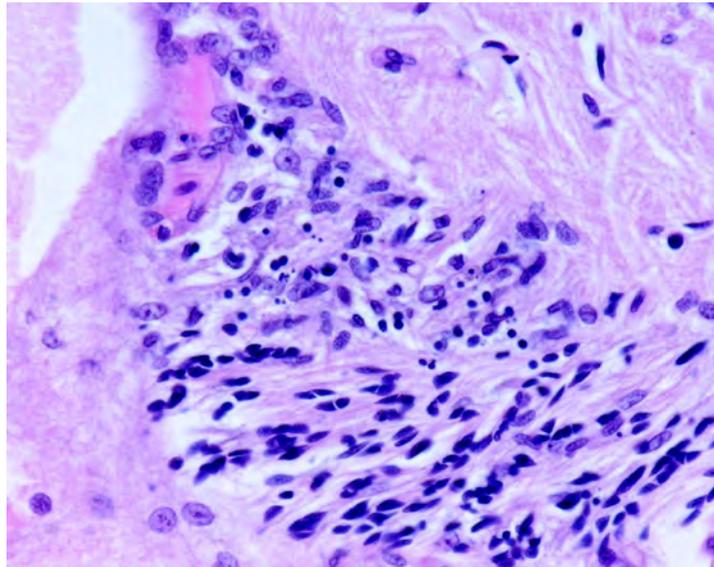


Figure 4.11. A Typical nerve chord lesion from *P. monodon*, x 400.

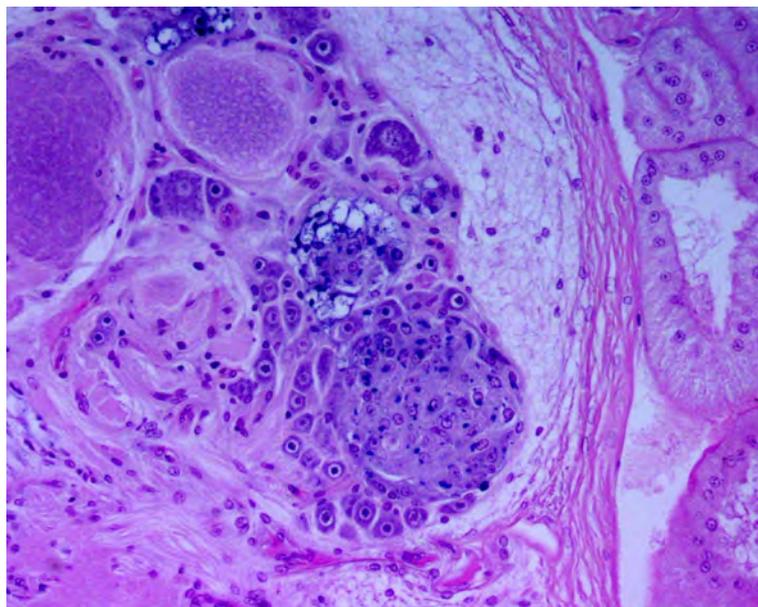


Figure 4.11. B: Typical nerve chord lesion from *P. monodon*, x 200.

CHAPTER 5. DISCUSSION

White Spot Syndrome Virus survey

White Spot Syndrome Virus is an exotic prawn virus of uncertain affinity that is associated with high mortality accompanied by white spots on the carapace of moribund prawns. It has a characteristic histopathology. Tissues of mesodermal and ectodermal origin have areas of necrosis associated with basophilic inclusions surrounded by marginated chromatin and, in early stages, resembling Cowdry “A” type inclusions (Kasornchandra *et al.* 1998). WSSV has never been reported from Australia but, following the diagnosis of WSSV in crustaceans fed imported affected animals at Darwin, a national survey to determine whether WSSV was present in Australian wild crustacean populations was undertaken during 2001.

The sampling regime of 30 animals from 30 sites used for the survey was based on advice from the CCEAD. However, there was difficulty in obtaining 30 “sites” in Western Australia due to the sparse settlement of the coastline and difficulty of access for fishing for crustaceans. In all 29 “sites” were sampled.

The issue of “false positive” results was somewhat problematic. Two crab samples tested inconsistently weakly positive for WSSV by nested PCR. This material also tested inconsistently weakly positive by nested PCR at AAHL (Geelong), although not with real-time PCR which was consistently negative.

There are four possible causes for these results:

- 1) Contamination of the sample with fragments of WSSV
- 2) Non-specific bands of the same weight as the expected WSSV fragment on the gel.
- 3) Presence of WSSV in very low titre in the sample.
- 4) Presence of a WSSV-like virus in Australian crabs, at very low titre.

Contamination was considered unlikely as similar “false positive” results were obtained from crabs (but not with prawns) in all of the laboratories testing for WSSV in Australia, including AAHL (Geelong). However, the PCR technique is known to be susceptible to contamination, even in laboratories where cleanliness is scrupulously observed (Navidi *et al.* 1992). Option two can be excluded, as sequence data from suspect samples confirmed WSSV sequence. The amplified fragments were, therefore, not “non-specific”.

Of the last two possible explanations, it may be considered unlikely that WSSV occurs in Australia since there is no epidemiological evidence for WSSV disease ever having occurred in Australian prawns. Published reports and other evidence from experiences overseas indicate that the virus does not occur naturally at extremely low titre in this manner.

The fourth possibility has not been eliminated. Given the reported close similarity of GAV/LOV to Yellow Head, the presence of a WSSV-like virus in crabs, perhaps in tissues other than epithelial tissue, cannot be discounted at this stage. However, the issue is one of extreme importance to Australia. Positive PCR by the OIE method is the accepted international standard for presence of WSSV. Testing of Australian exported product in overseas labs may well lead to similar “false positive” results being reported. If the OIE recommended two-step PCR does cross-react with something else and / or gives occasional “false positives”, there may be a need for some re-evaluation of published WSSV distribution data in the literature, particularly where occurrence is based on small sample sizes and a lack of duplicate samples.

Yellow Head and Gill Associated Virus (GAV)

Gill Associated Virus (GAV) and Yellow Head Virus (YHV) are both placed in the *Nidovirales* and are closely related (Cowley *et al.* 1999; Tang *et al.* 2002). Both are internationally notifiable. Of the two, YHV is considered exotic to Australia. It has a distinctive histopathology that can be used to derive a presumptive diagnosis (Wongteerasupaya *et al.* 1995). Classical yellow head lesions consist of areas of necrosis accompanied by small basophilic cytoplasmic inclusions, particularly in the lymphoid organ. These have never been seen in Western Australian prawns and testing of prawns by nested and real-time PCR has also failed to detect any trace of YHV.

In Australia, GAV occurs in Queensland and New South Wales. It is a complex virus manifesting in two forms. Lymphoid organ virus is commonly present in *P. monodon* but causes no mortality while gill associated virus can cause high mortality but presents gross signs (reddening, lethargy and mortality) and lesions in tissues that differ from those for YHV (Spann *et al.* 1997). GAV causes disorganization and loss of structure in the lymphoid organ, but without the nuclear hypertrophy, vacuolization and pyknosis seen in LOV. Gills of GAV affected prawns show loss of cuticle and fusion of gill filament tips and general necrosis (Spann *et al.* 1997). Comparison of DNA sequences has shown that LOV and GAV are the same virus (Cowley *et al.* 1999, 2000). GAV is transmitted vertically (Cowley *et al.* 2002a).

Histopathology consistent with GAV has not been seen in this survey. In addition, GAV has not been detected by molecular methods in prawns from Western Australia, and Western Australia is therefore considered to be free of GAV.

Monodon Baculovirus-like virus (MBV-like virus)

Monodon baculovirus is an occluded baculovirus believed to be a complex of several related strains infecting penaeids in the Mediterranean and Indo-Pacific regions, including Australia.

Lester *et al.* (1987) described an MBV-like virus from *M. latisulcatus* in New South Wales, Australia. MBV also exists in *P. monodon* hatcheries and grow-out ponds; and in wild *P. merguensis* in Queensland (Dobrovsky *et al.* 1988). An MBV-like baculovirus was reported from *Metapenaeus bennettiae* from Queensland by Spann & Lester (1996) and named by them as “Bennettiae baculovirus” (BBV). BBV has similar histopathology to MBV, however, it does not react to MBV specific DNA probes and there are differences in the relative proportions of the virions at the ultrastructural level (Table 5.1).

Table 5.1. Comparison of measurements of MBV and MBV-like baculovirus with the MBV-like virus from Western Australia.

Virus	Enveloped virion (nm)	Nucleocapsid (nm)	Authority
BBV	300-387 x 54-56	n/a	Spann & Lester (1996)
MBV from Taiwan	265-282 nm x 68-77	250-269 x 62-68	Mari <i>et al.</i> (1993)
MBV	324 x 75	246 x 42 nm	Johnston & Lightner (1988)
MBV from Queensland	n/a	260-300 nm x 45-52	Doubrovsky <i>et al.</i> (1988)
MBV from Queensland	325 x 70	295-320 x 44	Vickers <i>et al.</i> (2000)
MBV-like from Western Australia	270- 319 x 41-55 nm.	205-286 x 29-35	This study

Baculovirus nucleocapsids from an endeavour prawn (*M. endeavouri*) taken in Exmouth Gulf in November 1999 were histologically and ultrastructurally similar to MBV but the virus was smaller than both classical MBV and BBV. The Queensland MBV reacts to MBV-specific DNA probe according to Spann & Lester (1996), however, the Western Australian form did not react to the DiagXotics MBV ISH probes though all controls worked as expected. This may be because the tissues tested in our study had been fixed for too long in the initial treatment and were therefore not suitable for testing with the MBV kit (which is designed for fresh tissue). Alternatively, the MBV-like inclusions seen in WA prawns may be a different strain of MBV that is not detectable with the DiagXotics kit. Further testing on fresh material is clearly required.

Monodon baculovirus-like inclusions were relatively common in *M. endeavouri* and in hatchery produced *P. esculentus* post-larvae, but were rare in *P. esculentus* adults. Indeed, the infection in *M. endeavouri* was so ubiquitous as to suggest that the virus has been present and equilibrating with that species for a long time, and that *M. endeavouri* are the main reservoir of infection in the environment. In contrast, the infection in other species has much lower prevalence.

MBV-like virus was associated with a minor mortality problem in a pilot production run of juvenile *P. esculentus*, although losses (about 50%) were within normal hatchery limits and could not necessarily be attributed to the virus. Some batches showed severe infections with up to 100% prevalence whilst other batches were much less affected. Batch losses correlated very roughly with prevalence of infection, although there were clearly other unidentified factors contributing as well. Serial examinations suggested that the juveniles were highly susceptible to infection, and possibly mortality, up to about PL₁₀, but beyond PL₂₀ the juveniles appeared to lose their susceptibility to infection.

Chen *et al.* 1992 suggested that MBV occurs through the oral route through contaminated faeces, and that washing of fertilised eggs could prevent infection of post-larvae. Subsequent batches of prawn eggs were washed in the hatchery, and MBV-like inclusions have not recurred.

Although the PL samples did not provide evidence of a causal role of MBV-like virus in mortalities, they did show unequivocal evidence that *P. esculentus* are susceptible to natural infection with an MBV-like virus. The older animals collected with the P-99-4005 submission, on the other hand, showed no evidence of infection, despite living in close proximity to endeavour prawns with a prevalence of 72%. It is possible that susceptibility to infection with this virus is highly age-dependent in *P. esculentus*.

Prevalence data for MBV-like virus must be interpreted with caution as prevalence of MBV in the Philippines is known to be seasonal, ranging in wild and cultured stocks of *P. monodon* from 100% in February to 73% in July (Lightner *et al.* 1992b). Species apparently uninfected in this study included *P. monodon* and coral prawns (*Metapenaeopsis* spp.). MBV is a disease primarily of post-larvae and juvenile prawns, and the lack of evidence of inclusion bodies by histology in adult prawns does not mean that severe infections will not occur in culture. For example, Turnbull *et al.* (1994) reported that *F. merguensis* and *Metapenaeus* spp. cultured in north-east Sumatera, Indonesia, were not affected by MBV, yet other reports have been published of MBV in *P. monodon* (Lightner & Redman 1981) and in *F. merguensis* (Dobrovsky *et al.* 1988; and this study).

Dual infections with both MBV-like virus and HPV were commonly observed in *F. merguensis*. This is consistent with published records linking HPV with multiple infections (Lightner *et al.* 1992b; Turnbull *et al.* 1994).

Hepatopancreatic Parvo-like Virus (HPV)

HPV is found in Asia as well as in the Middle East, Africa and the American continent. It also occurs in cultured and wild penaeids in Australia (Paynter *et al.* 1985; Roubal *et al.* 1989).

HPV was observed both in the hepatopancreas and in the midgut of *F. merguensis*. It was not seen in *P. monodon*, *M. endeavouri* or coral prawns (*Metapenaeopsis* spp.). However, as with MBV-like virus, the negative findings are of little significance since HPV has previously been reported from *P. monodon* in Australia (Owens 1997) and may occur at low prevalence in the other species. The staining characteristic of HPV with H&E stains is known to be variable, from basophilic through to eosinophilic for inclusion bodies early in their development (Lightner *et al.* 1992b; Lightner & Redman 1992) and this was seen during our survey. HPV causes stunting in *P. monodon* farms in Thailand (Flegel *et al.* 1999).

Australian prawn viruses, other than GAV, not seen in this study

“Bennettiae baculovirus” was reported from *Metapenaeus bennettiae* from Queensland by Spann & Lester (1996) and has similar histopathology to MBV. See the discussion under Yellow Head Virus and Gill Associated Virus (Page 69).

IHHNV-like virus was reported from Queensland in a hybrid cross (*P. monodon*/*P. esculentus* by Owens *et al.* (1992b) who described how gill samples reacted to an IHHNV ELISA, giving values of 38 to 71% of the intensity of known IHHNV positive control. This proved to be an initial misidentification of the very closely related lymphoid parvo-like virus previously described by Owens *et al.* (1991). The affected host tissue does not react to the DiagXotics ISH for IHHNV (Owens 1997; Anderson and Owens 2001) and it is referred to in recent papers as Lymphoidal Parvo-like Virus. This virus has only observed in Australia in cultured *P. monodon* and *P. merguensis* (Munday & Owens 1998). There is no evidence,

based on histology, for the occurrence of LPV in Western Australia. IHNV is considered exotic to Australia (Herfort & Rawlin 1999)².

Penaeid Haemocytic Rod-shaped Virus was detected in the same hybrid shrimps infected with LPV (Owens *et al.* 1992b; Owens 1993; Munday & Owens 1998). The virus appears to infect fixed phagocytes and circulating haemocytes in the gills and lesions closely resemble those of White Spot Syndrome Virus (Munday & Owens 1998). The rod – shaped virions are 588 x 119 nm with longer virions up to 888 nm. There is no evidence, based on histology, for the occurrence of this virus in Western Australia.

Baculoviral Midgut Gland Necrosis Virus (BMNV) is an unclassified non-occluded bacilliform virus infecting prawns in Asia, principally post-larvae. Lightner (1996) reported this was present in Australia but no other information was given and it is currently considered exotic to Australia (Herfort & Rawlin 1999).

Spawner Mortality Virus (SMV) is listed by the OIE among “other significant diseases” of Crustacea. There is no specific histopathology associated with this virus and there are no diagnostic tests available (as at February 2003) though one is under development. It is therefore not possible to determine the status of Spawner Mortality Virus in WA.

The story of Spawner Mortality Virus began with a paper by Fraser & Owens (1996) which reported that wild-caught breeding prawns (*P. monodon*) in Townsville, Australia, were diseased with a red coloured appearance and red faeces. The associated histological lesions, including “large areas of necrosis” in the hepatopancreas, were reported to be “non-specific and easily confused with bacteraemic prawns”. The authors also reported that two of three affected prawns examined by electron microscopy showed 20 nm particles (which they termed SMV) but the lack of particles in the third prawn was not explained in the paper. Attempts by the authors to isolate virus particles on caesium chloride gradients were reported as unsuccessful, however, filtered extract of prawns was lethal to inoculated prawns so Fraser & Owens (1996) inferred that a lethal virus was present. In subsequent infection trials, also reported in their 1996 paper, deaths of control animals as well as trial animals were recorded. The deaths of the control animals were discounted as being due to SMV because “no lesions were evident” – in other words, non-specific lesions, including hepatopancreatic necrosis, was a characteristic of the infection by “SMV” in 1996. The conclusion reached by the authors that the “red disease” seen in prawns was associated with the 20 nm particles observed by electron microscopy was, at best, tenuous.

Further work followed. Owens *et al.* (1998) reported up to 80% mortality in 10-15 g *P. monodon* prawns in grow-out ponds. This they described as Mid Crop Mortality Syndrome (MCMS). Bioassays indicated that the mortalities were of viral aetiology. This time isolation of virus particles on caesium chloride gradients was successful and the authors reported a number of light diffracting bands. Of these, “one of the bands that appeared more commonly” and consisted of 20-25 nm particles was used to develop a DNA probe. The contents of the other bands were not described. The inference from the paper, however, is that the viral DNA from which the probe was developed belonged to one of at least two viruses present in the CsCl gradients and that three viruses may be involved in the MCMS (Owens *et al.* 1998).

The mortalities observed in Queensland prawns and ascribed to SMV in the Owens *et al.* (1998) paper produced no histopathognomonic lesions, in contrast to the paper of Fraser &

² IHNV was reported to occur in Australia in February 2004, as this document went to press. The Australian strain of IHNV does not react to the conventional OIE primer set.

Owens (1996). Red colour and the presence of red faeces, a specific characteristic of SMV in the 1996 paper, are not mentioned. The conclusion by Owens *et al.* (1998) that the 1996 and 1998 material were both “SMV” was based on tissue from both samples being gene-probe positive – despite the differences in the histopathology and the recognition that multiple viruses were present in at least the 1998 material. By contrast, in a separate paper, Munday & Owens (1998) described the histopathology associated with SMV as “non-specific consisting of deposits of refractile eosinophilic granular material under the cuticle and around the capsule of the digestive gland, haemocytic enteritis and hypertrophied nuclei with marginated chromatin in cardiac and skeletal muscle cells”. The status of the samples with respect to the virus GAV/LOV, common in *P. monodon* in Queensland and known to be associated with MCMS since the mid 1990’s (Spann & Lester 1997; Anderson & Owens 2001), is not explored in any of the papers, though Owens & McElnea (2000) did comment that MCMS was “a mixture of SMV and other pathogens, particularly, gill associated virus”.

A paper by Owens *et al.* (2003) using data from 38 ponds, attributed 6.7% of mortalities to SMV, but no attempt was reported to test for concurrent GAV infections in the trial ponds, or for the presence of Mourilyan virus (MoV). The role of GAV was acknowledged in their discussion with the sentence “Infection with lymphoid organ virus (Spann *et al.* 1995), the senior synonym of gill-associated virus (Spann *et al.* 1997), which is another component of MCMS is also likely to contribute to these losses”.

It has never been established in the published literature that the putative virus (SMV), detectable only by molecular diagnostic methods, is the sole cause of the prawn mortalities in Queensland prawns. Indeed, a more detailed study by Anderson & Owens (2001) concluded (their page 14) that Spawner Mortality Virus “is not always necessary for MCMS epizootics”. That study was not referred to by Owens *et al.* (2003).

The DNA probe developed at James Cook University and used to characterize SMV has been provisionally patented and was not available to us during our project. This makes reporting of SMV by positive gene probe impossible. In addition, the putative virus described as SMV is claimed to be one of a relatively large number of parvoviruses in prawns (based on size and on the unpublished DNA sequences (Anderson & Owens 2001). This makes diagnosis based on electron microscopy somewhat problematical.

Mourilyan virus (MoV) was also first isolated as DNA from Australian *P. monodon* and subsequently identified on electron micrographs (Cowley *et al.* 2002b). Initial reports associated it with nerve chord lesions, but subsequent work has indicated that this may not be the case. A commercial PCR kit is now available to test for this virus.

In summary:

- SMV (and MoV) are products of gene probe technology – based on gene probes created out of a DNA soup. A positive reaction to DNA tells nothing about the viability of the agent or its involvement in disease, SMV gene-probe positive cells in a range of host crustaceans are histologically normal.
- No disease has been attributed to SMV, yet it is now an internationally reportable disease. The observed disease signs associated with recovery of SMV viral DNA are different in the papers cited. The authors attribute the mortalities in the 1998 paper to MCMS, itself an ill-defined syndrome and subsequent work (Anderson & Owens 2001) has questioned the involvement of SMV in the syndrome.
- The presence of 20 –25 nm virus-like particles by electron microscopy is not sufficient to determine if the disease is SMV since there are many viruses of this size.

- Neither GAV, MoV nor SMV have been purified which makes any statement about the pathology associated with specific viruses, or their role in MCMS somewhat problematic.

An FRDC project entitled “Development of Diagnostic Capability for Priority Aquatic Diseases of National Significance: Spawner-Isolated Mortality Virus”, to develop and make available a diagnostic test for SMV, is now underway and, once the diagnostic test is available this will enable researchers to verify the pathology associated with the virus.

Rickettsial diseases

No rickettsial or chlamydial agents were seen during the survey and none have been reported in the literature from Australia (Kahn 1998).

Bacterial diseases

Bacterial diseases, especially those associated with *Vibrio* species, are ubiquitous in shrimp and prawns, and Australia is no exception. *Vibrio harveyi*, together with an unidentified flexed gram negative bacteria and a fungus, have been isolated from *P. esculentus* in Queensland (Owens *et al.* 1992a). Because the majority of the samples examined were wild caught prawns the incidence of bacterial disease was relatively low. Vibriosis occurs worldwide in marine crustacean culture systems, to the point where bacterial infections are often dismissed as “vibriosis” without any attempt at isolation and identification of the bacterial species present. Some strains appear to be primary pathogens, including *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* (Lavilla-Pitogo *et al.* 1990; Nash *et al.* 1992; Costa *et al.* 1998; Teunissen *et al.* 1998). All of these species are ubiquitous in Australia. Bacteria, including vibrios, are part of the natural microflora of the pond sediment, increasing significantly during the growout season, and often become opportunistic pathogens when the animals are stressed through poor water quality, temperature changes, or other factors (Le Moullac *et al.* 1998; Smith 1998).

Whatever the species of bacteria, or the tissue affected, there is always a marked cellular response by prawns to the presence of bacteria (Chapter 3) resulting in focal inflammation, phagocytosis encapsulation and deposition of melanin as a result of the initiation of the prophenyl oxidase defence mechanism. The presence of mild acute and otherwise unexplained inflammation observed in various tissues including nerve chord, muscle tissue, antennal gland, heart and cuticular epithelium in *P. monodon* was often attributable to bacterial disease. Many of these prawns had been kept in captivity for various periods before necropsy.

Bacterial shell disease, characterized by pitting and focal melanisation under and within the cuticle, is caused by bacterial and/or fungal attack on the chitin exoskeleton. This was not seen in this survey, but does occur in Australia on *P. esculentus* (Paynter 1989, Owens *et al.* 1992a). Haemocytic enteritis was observed in a *P. esculentus* from Shark Bay in 1999 which may have had a bacterial origin, though haemocytic enteritis has also been associated with injection of blue-green algae (Lightner 1978; Lightner *et al.* 1987) as well as bacteria (Lightner 1996).

Fungal diseases

Fungal diseases (such as *Fusarium* sp. and *Lagenidium* sp.) were not seen during this survey. Undoubtedly that will change as hatchery and pond culture proliferates in Western Australia. An outbreak of mortality in *P. esculentus* broodstock due to a mixed bacterial and fungal infection was described by Owens *et al.* (1992a). The septate branching fungus provoked a

marked haemocytic inflammatory response and associated melanisation of the affected cuticle and muscle. The fungus was accompanied, on the surface of the cuticle, by filamentous bacteria, and pure cultures of *Vibrio harveyi* were isolated from the haemolymph.

Microsporidiosis

Microsporidians are the most common parasites in Crustacea (Meyers 1990) so the lack of microsporidians in the samples is noteworthy. However, this may simply be a reflection of the sampling strategy since infected prawns tend to inhabit areas where trawlers do not fish (Overstreet 1973). Microsporidians tentatively identified as *Ameson* sp. do occur in *F. merguensis* and *P. semisulcatus* at Melville Island in the north Kimberley, Western Australia (Owens & Glazebrook 1988).

Haplosporidiosis

There are no published records of haplosporidians in Australian prawns though there is an unpublished record of their occurrence in *P. monodon* in Queensland (Kahn 1998). Haplosporidians have already been recorded in prawns from Indonesia and the Philippines (Lightner 1996) so their presence in Western Australia is unsurprising.. Haplosporidan infections have been associated with slow growth of shrimp in commercial ponds and have the potential to become a significant disease problem (Dr C.V. Mohan, Thailand, pers. comm.).

Spores have never been demonstrated for these haplosporidian parasites in prawns and crabs (Perkins 1989) and they are assigned to the Haplosporidia based on the ultrastructural demonstration of haplosporosomes in the morphologically similar parasite found in the hepatopancreas of the shrimp *P. vannemai* by Dykova *et al.* (1988). An ultrastructural study of the Australian parasite is therefore warranted.

Ciliates and flagellates

Ciliates were not seen during the survey of wild caught prawns. However, ciliates are a problem for prawns in culture, and unidentified ciliates have been associated with culture of *P. monodon* PL's in Western Australia. Paynter (1989) recorded the presence of peritrich ciliates as fouling on *M. latisulcatus* in New South Wales. Lightner (1988) reported that *Parauronema* sp. , *Paranophrys* sp. and the flagellate *Leptomonas* sp. have all been implicated with infections of post-larvae. It is likely that most ciliate infections of prawns are either overlooked or treated without being reported in the literature. Paynter (1989) noted that the presence of peritrich ciliates fouling prawns could be used as an indication of the level of organic load in the water, since they thrive in conditions of low water exchange and high organic loading.

Flagellates are not regarded as serious pathogens of prawns but rather as symbionts or opportunistic pathogens (Meyers 1990). *Cryptobia*-like flagellates are not uncommon in fish but have not been recorded before from prawns.

Gregarines

Not previously reported from prawns in Australia, probably because of their lack of significance. Gregarines have been associated with crustaceans on every continent, including Australia (Kahn 1998). Gregarines have an intermediate host, usually a polychaete or a mollusc, and infection is by ingestion. The life cycle of the gregarines is usually short. Overstreet (1973) reported that brown shrimp (*P. aztecus*) kept in aquaria lost their gregarine

infections after 8 days. The finding of what appear to be inclusion bodies in gregarines has not previously been reported, and should be investigated further.

Miscellaneous metazoan parasites

Prawns and shrimps act as intermediate hosts for many parasites. Unidentified digenean metacercaria, trypanorhynch cestode plerocercoids, acanthocephalans and nematode larvae are therefore not uncommon in prawns and shrimps, including in nervous tissue. Their effect on the host is unknown and they are generally regarded as innocuous (Meyers 1999).

HISTOPATHOLOGY OF UNKNOWN AETIOLOGY

Crystalline deposits have been reported from the antennal gland of *Cherax quadricarinatus* (Edgerton 2000) and also occur in *Cherax tenuimanus* (pers. obs.).

Crystalline intranuclear inclusion bodies

The inclusion body-like polyhedral crystals look very much like virus inclusion bodies, but they have been reported from Australian penaeids before. Paynter *et al.* 1985 described “HPV-like” angular, intranuclear “cubic basophilic inclusions” from *M. bennettiae* digestive gland cells. These affected nuclei were described as being without the characteristic nucleolus present as a “signet ring” on the side of the developing inclusion body. They reported that the crystalline bodies did not stain with Feulgen stain and were attributed by them to a toxic disease syndrome associated with blue-green algae as described by Lightner & Redman (1984). However, the crystalline proteinaceous inclusions described by Lightner & Redman (1984) were described as eosinophilic and the marked haemocytic enteritis associated with blue-green algal syndrome by Lightner & Redman (1984) was not mentioned by Paynter *et al.* (1985). A subsequent paper by Lester *et al.* (1986) described light to dark purple staining (in H&E stain) crystals in the nuclei of the hepatopancreatic cells of *P. esculentus*. With an electron microscope these were shown to have a lattice structure, interpreted to be protein, as described by Lightner and Redman (1984).

Crystalline deposits in Antennal gland

These were only seen in *M. latisulcatus* from Shark Bay, but have also been seen in antennal glands from freshwater crayfish (*Cherax albidus*) in Western Australia. They are believed to have an environmental aetiology.

Eosinophilic Virus-like Inclusions

These large eosinophilic virus-like inclusions were commonly observed. The depth of stain colour varied from pale to bright and in cases were similar to HPV. It was not possible to determine definitively, from a histological study alone, whether all eosinophilic virus-like inclusions were representative of the same structures, or even whether the cellular location was cytoplasmic or nuclear. The inclusions may be of reovirus origin (if cytoplasmic) or a variation of HPV-like or MBV-like virus inclusions (if nuclear), or neither of the above. Attempts to examine the eosinophilic virus-like inclusions under the electron microscope did not reveal virus-like particles in the one animal examined, but the tissue was very poorly preserved. It may well be that the eosinophilic “inclusion” is just an artefact resulting from condensed protein. Some of the prawns showing these eosinophilic virus-like inclusions were tested for HPV using a commercial HPV ISH kit, and were non-reactive with the HPV probe, indicating that these inclusions are not HPV.

Small eosinophilic inclusion-like bodies

The inclusions observed in tegumental glands of four *P. monodon* were also observed in the nerve chords of two of those cases. It may be that an unknown virus contributes to the causation of inflammatory nerve chord disease resulting in these small eosinophilic bodies in cells or they could represent autolytic change (see above discussion for eosinophilic virus-like inclusions).

Lymphoid organ spheroids and ectopic spheroids

Lymphoid organ spheroids have been reported in *Penaeus monodon* from Queensland (Cowley *et al.* 2002a).

Lymphoid organ spheroids are commonly associated with viral infection (Hasson *et al.* 1999). However, in the current study, the lymphoid organs of all species except endeavour prawns were commonly affected by spheroid formation, which ranged from mild to severe. In some cases the spheroid cells contained small basophilic inclusions in the cytoplasm that was suggestive of a viral infection, however EM examination consistently failed to find virus particles within the “inclusions”. Their ultrastructure under EM was suggestive of non-specific phagocytic lysosomes. The lymphoid organ is thought to function as a haemolymph filter (Martin *et al.* 1987; Bell & Lightner 1988). Anggraeni & Owens (reported in Hasson *et al.* 1999) showed that spheroids were of haemocyte origin and Hasson *et al.* (1999) experimentally induced spheroid formation associated with Taura syndrome virus. They suggested that spheroids (both in the lymphoid organ and elsewhere) are part of the chronic inflammatory response and represent a form of aggregation associated with the phagocytic process and are involved in trapping and neutralizing unwanted particles (see Chapter 2). The lesions we saw were very similar to those described by Soledad *et al.* (1999) in white prawns in Mexico. That study found the prevalence of significant spheroids in wild-caught adults was 20% and 37.5% respectively for two independent samplings, and that an increased hatchery holding time, with respect to the second sample, was the most likely explanation of the increase in prevalence.

In our survey, the average severity of the syndrome as observed in any sample group tended to vary with the species and the geographical location. For example, though *P. monodon* broodstock samples received from Broome TAFE were equivalent in every respect other than the timing of their submission, sample AS-01-4005 (from Joseph Bonaparte Gulf in November 2001) showed much more severe spheroid formation and with abundant basophilic “inclusions” than either AS-00-2748 samples or AS-01-2971 samples, which showed milder spheroid formation with very few “inclusions”. The *P. monodon* submitted directly (AS-01-1535) showed virtually no spheroid formation at all.

Although at least six viral infections are commonly reported to be associated with lymphoid organ spheroids (Hasson *et al.* 1999), none of the viruses observed in this study were correlated with spheroid formation. Indeed, numerous banana prawns (*F. merguensis*) from the De Grey river area in May 2001 were observed to have high infections with HPV-like virus or MBV-like virus or both, but with no changes in the lymphoid organ at all.

Ectopic spheroids were also commonly observed during our study and were correlated with the occurrence of lymphoid organ spheroids in *P. monodon* and *M. endeavouri*. This pattern was previously observed by Lightner *et al.* (1987) and associated with chronic inflammatory infections. Ectopic spheroids have been associated with tegumental glands (Hasson *et al.* 1999), but this relationship was not observed in our study. Hasson *et al.* speculated that lymphoid organ spheroids and ectopic spheroids formed in the same manner and were the same phagocytic cell type and that they were a defensive response to foreign bodies which

were too small to elicit an encapsulation reaction. Our study would concur with that view. The ubiquitous nature and the epidemiological pattern of spheroid formation suggested that they were associated with either a multifactorial cause or an equally common pathogen such as *Vibrio* bacteria. A bacterial cause with a significant component of chronic stress of the host was the most likely explanation of the lymphoid organ spheroids observed across all prawn species examined in this study.

Nerve chord syndrome

Gut and nerve syndrome of Lightner (1988) is diagnosed by histological demonstration of a thick eosinophilic PAS positive amorphous layer representing a marked hypertrophy of the midgut mucosal basal membrane as well as a hyperplasia of the epineurium covering the ventral nerve chord into multiple repeating layers between which are granulocytes. This was also reported by Owens (1997) in wild banana shrimps (*F. merguensis*) and *Marsupenaeus japonicus* from Queensland. The epineural proliferation was prominent but with substantial haemocytic (as opposed to granulocyte) infiltrates between the epineural layers (Munday & Owens 1998).

In the present study a separate nerve chord lesion was identified in which individual neurones were commonly observed degenerating and undergoing phagocytosis, followed by loss of the neurone and its replacement by infiltrating haemocytes and apparently the eventual formation of an ectopic spheroid. The association of this nerve lesion with the newly reported Mourilyan virus has not been explored. Mourilyan virus may be associated with gut and nerve syndrome (Cowley *et al.* 2002b).

Juvenile stages of an unidentified metazoan parasite were commonly found in the nerve chords. There was no evidence from either histology or statistical correlation for the parasites being causally associated with the nerve chord syndrome.

A third nerve-chord syndrome has recently been described in *P. monodon* from eastern Australia and is associated with GAV (Callinan *et al.* 2003; Callinan & Jiang 2003). The GAV associated syndrome results in mild to severe focal degeneration and necrosis of axons and their sheaths and apoptosis of associated glial cells in peripheral nerve fibres. Degeneration and necrosis of reticular cells and associated axons was also observed.

GAV has never been detected in WA prawns.

BENEFITS

The major beneficiaries of the project are identified as:

- Prawn farmers
- Prawn exporters
- Regulators
- Fish Health researchers

This survey provides, for the first time, an overview of the disease status of the major wildstock prawn fisheries in Western Australia. The data provide farmers and fish exporters with a “snapshot” of the health of the fishery upon which their business plans can be based. In particular, the lack of evidence for GAV/Yellowhead in WA prawns provides opportunity for export of *P. monodon* PL’s to the eastern States and overseas. However, it should be realised that prawns from WA are not “disease free” and there are lesions suggestive of a potentially unique virus fauna. These may pose a threat to eastern States populations. The apparent absence of GAV also provides regulators with evidence against which to block unrestricted movement of live prawns from the eastern States into WA.

The data also provide additional evidence for the zoogeographic barrier at Torres Strait (Owens 1990) and it is to be regretted that movements of prawns from Queensland into Northern Territory for aquaculture have effectively overcome this barrier at least for GAV.

INTELLECTUAL PROPERTY

No intellectual property has been generated.

FURTHER DEVELOPMENT

The visualisation, by histology, of lesions consistent with a viral aetiology in WA prawns (small eosinophilic virus-like inclusions; nerve syndrome) requires further work to determine if a virus is actually involved.

The acquisition of PCR and ISH technology to detect Mourilyan virus and Spawner Mortality Virus will allow us to test WA prawns for these new and emerging disease agents for which specific lesions have not yet been described.

Finally, surveillance of the populations from which these samples were taken will need to continue since there are known disease agents in the populations which were at such low prevalence that we did not detect them (Microsporidiosis) and there are likely to be other novel agents present which, for the same reasons, we also did not detect .

The existing development of hatchery technology in WA and the eventual development of growout farms will undoubtedly reveal disease problems – as has occurred in Queensland and New South Wales.

STAFF

Staff in the following institutions contributed to this project either by providing technical assistance or by contributing to the text.

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CONCLUSIONS

- As per Objective 1, the project has acquired information on the disease status of the commercial prawn species on the northwest shelf and Shark Bay. The information collected indicates that the prawns are free of GAV but are exposed to MBV-like virus and HPV (these two viruses are already known from Australia).
- All of the testing of prawns and crabs in WA proved negative for WSSV.
- These data are of use in documenting Australia's prawn disease status both for import and export purposes.
- The project has not negated the hypothesis that the prawn stocks to the west of the Torres Strait have a distinctive parasite fauna and thus controls on movement of prawns between the western Australia and other States and the Northern Territory should either remain or be strengthened.
- As per Objective 2, the project has provided a greater understanding of the histopathology of prawns in Australia. Syndromes and lesions encountered during this study are described in the report.
- The project has resulted in the development of closer ties between the laboratory in Western Australia and the CSIRO Indooroopilly.
- Freedom from GAV by Western Australian sourced *P. monodon* is of great scientific interest in that this species is affected by a number of viruses in combination with GAV in both Queensland and New South Wales. The existence of a GAV-free stock may be of use to better understand the role of GAV in Mid Crop Mortality Syndrome and also may assist in attempts to close the life-cycle of *P. monodon* in Australia.
- The issue of WSSV 'false positives' by PCR needs further attention, since testing of Australian product overseas using the OIE method of Lo *et al.* (1996, 1997) may well result in false claims that the virus exists in Australian waters. It also raises the issue of whether literature reports of WSSV overseas, based on the Lo method, are in fact true positives.

CHAPTER 6: REFERENCES

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