The diagnosis and prevention of the mid-crop mortality syndrome of pond-reared black tiger prawns (*Penaeus monodon*)

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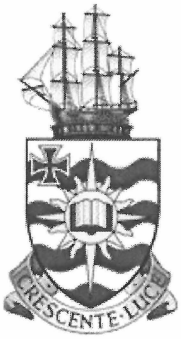
Queensland
Government
Department of
Primary Industries



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NON TECHNICAL SUMMARY

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OBJECTIVES:

1. To refine the detection system (hybridisation and PCR) to ensure the best sensitivity and specificity for a test to detect the MCMS agent.
2. To clearly identify the risk factors which aggravate the expression of clinical MCMS, including the role of marine blue-green algae.
3. To establish a model screening program which allows hatchery and farm production with virus-free prawns.
4. To determine a cost effective, environmentally sensitive disinfection program to eradicate the MCMS agent from farms and hatcheries which will be a model for future disease emergencies.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

The project has created knowledge which has led to the outcome of better health management on Australian prawn farms. Changes to farm practices have occurred where whole-farm destocking and pond disinfection are applied as a hygiene practice following a season where crop declines or failures are seen. During, and subsequent to, the project prawn farmers now understand that infectious disease can affect cultured marine prawns in earthen ponds. As a consequence many farmers will submit sick prawns for diagnostic analysis soon after stressed prawns are seen in ponds, not assuming the problems are due to pond environment factors.

The project has contributed to the recovery of the Queensland prawn farm industry from the low production seen in the 1995/96 season of 1,294 tonnes (worth $28.2m) to 1855 tonnes (worth $32.4m) reported in 1998/99. A similar outcome is reflected when Australian marine prawn production is considered; from a low of 1,400 tonnes in 1994/95 to 2,059 tonnes of production in 1997/98.

The project has shown that a virus associated with Mid-Crop Mortality Syndrome is passed from breeders to their progeny. The project has created the knowledge of a detection test and that application of that test can be used by hatcheries to reduce the effect of the virus on productivity in the hatchery and farms.

Need

The Mid-Crop Mortality Syndrome (MCMS) outbreaks on Queensland prawn farms, which made this, and other research projects, necessary, appeared to have peaked in the 1996/97 season. Combined losses to prawn aquaculture were in excess of $5m. Initial investigations on Mid-Crop Mortality Syndrome found at least four different viruses (identified by electron microscopy) were present in diseased prawns. Initial step-wise purification to prepare refined suspensions of viruses at James Cook University identified a small parvo-like virus (now thought to be related to parvo and denso viruses) naming it Spawner-isolated Mortality Virus (SMV). Another virus, also considered to be involved in Mid-Crop Mortality Syndrome, was identified by Dr K. Spann (CSIRO Livestock Industries) and was named Gill-associated Virus (GAV). Once the infectious, viral nature of the disease syndrome was recognised it became clear a sensitive detection test was required to study the disease and to have tools that would allow effective management of Mid-Crop Mortality Syndrome. This project focused on developing and refining the molecular detection tests for Spawner-isolated Mortality Virus. Often it is not possible to exclude infectious agents from production systems and management of the disease needs to take a holistic approach. There was a need to investigate risk factors that could result in the initiation of disease outbreaks. Similarly it was important to evaluate methods to eradicate the virus(es) from farms after outbreaks and to screen out the virus from the broodstock used in prawn hatcheries. By the end of the 1996/97 season, Mid-Crop Mortality Syndrome outbreaks typically occurred close to pond harvest, not just mid-crop. Mid-Crop Mortality Syndrome has not caused widespread significant problems since 1997/98.

Detection Tests

An accurate and sensitive detection test (Test A) has been developed. Test A is based on molecular technology utilising a precise molecular key for virus recognition (primers) and the polymerase chain reaction (PCR) to amplify a molecular product so that a positive test result can be easily detected in the laboratory. A further test, Test A-2, was then developed to be applied after Test A to confirm a positive result was due to the detection of Spawner-isolated Mortality Virus. This set of tests were shown to be very sensitive, detecting as few as 100 viruses in a sample. Methods to use Test A/A-2 were refined to specifically detect Spawner-isolated Mortality Virus in adult prawn faeces. Targeting faeces as the sample was desirable as it allows repeated sampling of broodstock without damaging the animal. Test A/A-2 cannot yet be used to detect Spawner-isolated Mortality Virus in prawn fry (post-larvae) because of interference with post-larval tissue components. A second type of molecular test (an *in situ* hybridisation [ISH]), Test I was also developed to detect Spawner-isolated Mortality Virus directly in preserved prawn tissues. Test A/A-2 and Test I have been used often during the project to show they are appropriate, accurate and useful.

Risk Factors

Analysis of preserved prawn tissues using the Test I-type detection test (ISH tests) for both Spawner-isolated Mortality Virus and Gill-associated Virus (provided by K. Spann, CSIRO Livestock Industries) found that neither virus was always present in Mid-Crop Mortality Syndrome-diseased prawns. On analysis of archival prawn tissues collected from prawn farm disease investigations, it was found that both Spawner-isolated Mortality Virus and Gill-associated Virus have been infecting prawns since, at least, 1992. Imported commercial ISH detection test kits for exotic prawn viruses were used on diseased prawn tissues. Monodon

Baculovirus (MBV) and Hepatopancreatic Parvovirus (HPV) viral infections in Queensland prawns were the same as these viruses overseas (they gave a positive result). The results were negative for all other major exotic prawn viruses (these being White Spot Virus (WSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Taura Syndrome Virus (TSV), Yellowhead Virus (YHV) and *Baculovirus penaei* (BP)). The analysis of clinical records failed to identify any single, specific stressor that would initiate a Mid-Crop Mortality Syndrome outbreak. Specific research on cyanobacterial (blue-green algae) toxicity failed to find the cyanobacteria toxins had any effect on juvenile prawns. As a consequence it is still not clear why lethal infections occurred in 1994 then stopped causing problems by the 1998/99 season. We proposed a hypothesis, for Mid-Crop Mortality Syndrome to occur one or more viruses must have a critical level of prevalence in a pond population (and probably a critical intensity of infection in individual prawns) and that two or more different stressors need to act at the same time on that population of prawns.

Model Screening Program

As Spawner-isolated Mortality Virus originates in the wild-caught broodstock, Test A/A-2 has been used to test broodstock faeces in a commercial hatchery. Analysis of the survival of the progeny of these broodstock showed that 15% or more of the losses in ponds could be attributed to Spawner-isolated Mortality Virus infections. While further tracing of individual spawners and their progeny would be desirable, the elimination of broodstock carrying a Spawner-isolated Mortality Virus infection could well improve productivity in the Australian prawn farming industry. Screening of broodstock in commercial hatcheries appears to be a feasible strategy.

Disinfection Program

While considerable efforts to purify single viruses from Mid-Crop Mortality Syndrome diseased prawns were made we could not achieve this and use single virus suspensions for infection studies. As a result experimental infections used virus preparations made from diseased prawn carcases and probably contained a mixture of viruses. Mid-Crop Mortality Syndrome virus(es) are highly infectious. Very low doses (0.05ml of a 1:1,000,000 dilution of tissue) would cause lethal disease when injected. It was demonstrated that Mid-Crop Mortality Syndrome can be spread during an outbreak by healthy prawns eating infected prawn tissues and also via virus contaminated water. This indicates farms do need to consider movement of sick prawns (control scavenging birds) and water transfer (aerosol drift from paddle wheel aerators) when trying to control the spread of a Mid-Crop Mortality Syndrome outbreak. Experimental infections demonstrated that other prawn species were susceptible to Mid-Crop Mortality Syndrome by injection challenge, but that crabs were not. In practice, in on-farm outbreaks, this experimental susceptibility has not been seen. When more than one species of prawn is cultured at the same site, Mid-Crop Mortality Syndrome outbreaks in the Black Tiger prawn (*Penaeus monodon*) do not spread to Banana prawns (*P. merguiensis*) or Kuruma prawns (*P. japonicus*). Chlorine disinfection is highly effective for destroying the Mid-Crop Mortality Syndrome virus(es), even when entire prawn heads are used in experiments. Surprisingly desiccation (drying prawn heads in the sun) was not all that effective. Even after one week in the sun there was enough live virus(es) remaining to infect healthy prawns. It is clear the recommended farm clean-up procedures following viral disease on prawn farms (Bell and Lightner 1992; Dixon and Dorado 1997) will be effective for Mid-Crop Mortality Syndrome. One of the factors for the decline in Mid-Crop Mortality

Syndrome incidence in Queensland is probably due to the implementation of whole-farm destocking and pond disinfection undertaken on many of the affected prawn farms.

Further research to develop Test A/A-2 to reduce the time it takes to get a result is desirable. Further refinement of Test A/A-2 for use on post-larvae would aid viral screening prior to movement of prawn fry. Also, if the tests can be refined so that less skilled technicians can use them, their use would be more widespread and applicable to hatchery-level laboratories. Further research is required to understand the role of the different viruses, the role of multiple environmental stressors and the initiation of Mid-Crop Mortality Syndrome-like diseases.

Key words: Marine prawns, aquaculture, virus testing, disease prevention.

2. Background

In Australia the estimated farmed-prawn production in 1997/98 was 2,059 tonnes worth $35.4 million, growing from the 1,400 tonnes worth $21 million produced in 1994/95. Most of this production comes from farms north of Mackay on the east coast of Queensland. In the 1994/95 season a new disease was seen in farms in northern Queensland causing significant mortalities in pond-reared, juvenile black tiger prawns (*Penaeus monodon*). The disease now called Mid-Crop Mortality Syndrome (MCMS), initially only affected production in northern farms but appeared to affect farms throughout Queensland in the 1995/96 and 1996/97 seasons, with industry estimating losses over $5 million. Figure 1 illustrates the impact of MCMS (and the availability of PLs for pond stocking) on Queensland's farmed-prawn production. Note there was no effect on the production of *P. japonicus*. A syndrome named Monodon ganglioneuritis was described from a New South Wales prawn farm in 1999. It is possible that this is related to MCMS as it is thought the aetiology involves at least one of the viruses described from prawns with MCMS. MCMS seems unique to Australia. The literature does not describe similar conditions in any other countries which culture *P. monodon* eg., Indonesia, Thailand and the Philippines. Although a complex of pathogens associated with pond disease problems in the Philippines was shown to include one of the viruses (Spawner-isolated Mortality Virus) found in MCMS prawns in Queensland.

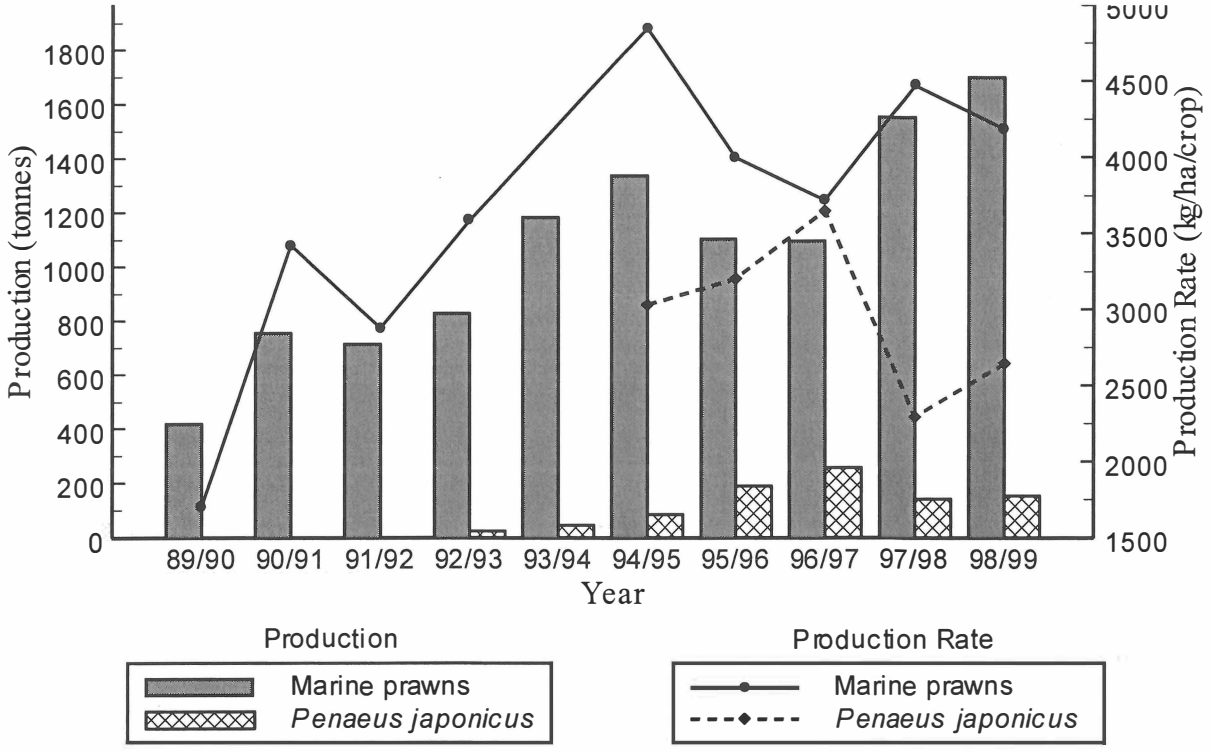

Figure 1: Queensland prawn farm production and production rate 1989/90 to 1998/99.

In the second half of 1994 several prawn farms in northern Queensland reported unusual mortalities of juvenile pond-reared black tiger prawns. The first reports coincided with a rapid warming of seawater temperatures in spring. Clinically, in affected ponds, sick prawns would usually appear after 3-3.5 months of pond culture or when they were greater than 12-

15 grams in size. Growth and survival prior to this would be normal and occasionally better. Feed consumption would fall suddenly, the algal bloom would often thin and a week later weak lethargic prawns with a slightly dark reddened body colour would appear at the pond edges. Diseased prawns would develop cuticular fouling, appendage erosion and shell disease. At times the pond experienced blue-green algae blooms at the time of pond mortalities. Mortalities would begin and continue at high levels until the pond was harvested. Increased water exchange and other changes in husbandry failed to alter the course of an outbreak.

Some of the affected farms reported a cycle of mortalities with peaks and troughs. The farms reporting disease were all well established with long experience in prawn farming. Not all farms were affected to the same extent, some only having one or two crops affected.

By September 1994 several farms had experienced repeated epizootics with different ponds affected. At this time, and increasingly so, an intensive investigation by scientists and pathologists from Queensland Department of Primary Industries, James Cook University (JCU), University of Western Sydney, CSIRO-Fisheries, and AIMS, and consultants was done through 1994 and 1995. A number of aspects were investigated, these included: pathology, microbiology, pond water quality, intake water quality, bloom composition, blue-green algae bloom, feed quality and pond management (I. Anderson, presented at the Australian Prawn Farmers Association Annual Conference, 27 July 1996).

The pathological findings were inconclusive. The only common finding was that some prawns had systemic bacterial infection, an infection secondary to stress. Only one of the many prawns examined, had gut damage due to consumption of blue-green algae. Changes in pond fertiliser schedules, water management and other investigations failed to identify any consistent factor or detectable water quality problem associated with disease outbreaks.

In April 1995 QDPI provided funding to support a scientist to do specific studies on water quality, marine blue-green algae and viruses. Dr P Muir was seconded from JCU to do this work. Dr L. Owens (JCU) duplicated viral transmission at the same time. This work demonstrated a virus (or viruses) was associated with diseased prawns and this virus(es) could cause death in experimental animals. This effect was demonstrated with infected material from four farms. Preliminary virus purification from MCMS affected prawns identified four virus-like particles under transmission electron microscopy. The inability to control outbreaks of disease with management to optimise pond conditions had serious implications on the future viability of prawn farming in Australia.

Later in 1995, several farms, not previously affected by MCMS, reported low level, chronic mortalities in over-wintered prawns. When water temperatures increased in September (1995) one of the original farms reported low level, chronic mortalities in all ponds held over the winter, even 8 gram prawns were affected. At that time six farms, all in north Queensland, have reported small losses. It is not clear if these are related to MCMS. There are no consistent pathological lesions in these prawns. These outbreaks may have be a different clinical expression of the MCMS virus(es). Since these initial crop failures, and coinciding with changes in farm management practices in northern Queensland, the number of farms affected by MCMS was limited to four (as assessed by submissions of diseased prawns to QDPI) in the 1996/97 season and two in 1997/98. Since that time there have been no reliable diagnosis of MCMS in Queensland prawn farms, although problems on one NSW prawn farm appear linked to MCMS virus(es).

The virus that had been purified at JCU was named as Spawner-isolated Mortality Virus (SMV) – named as such as it was first found from diseased spawners in a research facility. James Cook University provided one year of funding for a molecular biologist to develop a DNA probe or PCR detection test for the virus. QDPI provided, through the Queensland Fisheries Research Trust Fund, funds for a research assistant at JCU and QDPI until June 1996, $30 000 operating funds for JCU and $20 000 for OVL to carry out detection test development and further investigations into Mid-Crop Mortality Syndrome (MCMS). CSIRO-Fisheries contributed $10 000 to JCU to support the development of the detection test. The lack of any specific lesion that can be found histologically on a consistent basis prevents any attempts to detect MCMS, even in sick prawns. The development of a detection test was critical to any further understanding of MCMS and its control.

This project focused on the development of molecular detection tests for SMV. The SMV virus which is thought to be involved in causing MCMS has been isolated in pure form on a CsCl gradient. This is an absolute pre-requisite requirement for isolating the virus nucleic acid and subsequent production of a genetic probe and PCR system for specific detection of this virus. The purity of this preparation was very high, as judged by polyacrylamide electrophoresis of the sample. This technique demonstrated the presence of only 4 proteins by silver staining. Nucleic acid has been visualised by gel technique and is currently undergoing further characterisation. Since this is the first time that the virus has been purified, the virus has, in pure form, been injected into healthy prawns to verify that this virus is viable and causes the same symptoms as observed during previous transmission experiments. Another virus, also thought to be involved in MCMS, has been identified by Dr K. Spann, CSIRO Tropical Agriculture and was named Gill-associated Virus (GAV). The CSIRO team lead by Dr P. Walker undertook a CRC Aquaculture funded project to specifically develop molecular detection tests for GAV (and the non-pathogenic version of GAV named Lymphoid Organ Virus (LOV)).

3. Need

The current research is funded until June 1996. A sensitive detection test, based on a DNA probe for *in situ* hybridisation and a PCR detection system, to detect the virus in prawns and other material will be available at that time.

The research needs to continue so that the source of infection can be determined, batches of post-larvae or broodstock can be screened to give assurance to farms they are stocking ponds with virus-free prawns. Part of any future research will be the continued refinement of the DNA probe and detection test system to ensure optimal sensitivity and appropriate specificity. The virus incidence and susceptibility of other wild crustacea to the virus needs to be determined. This will provide further information on the possible sources of the virus. The effects of disinfectants and different farm clean-up procedures need to be evaluated. Ultimately a cost-effective process using a sensitive detection test and other procedures has to be developed so farms no longer suffer the mortalities now seen on northern Queensland prawn farms.

The Australian prawn aquaculture sector have no previous experience in industry-wide health testing programs. There is a need to establish such model programs for industry to best exploit export markets in the future so industry can provide assurance of a disease-free status for Australian product.

4. Objectives

1. To refine the detection system (hybridisation and PCR) to ensure the best sensitivity and specificity for a test to detect the MCMS agent.

2. To clearly identify the risk factors which aggravate the expression of clinical MCMS, including the role of marine blue-green algae.

3. To establish a model screening program which allows hatchery and farm production with virus-free prawns.

4. To determine a cost effective, environmentally sensitive disinfection program to eradicate the MCMS agent from farms and hatcheries which will be a model for future disease emergencies.

5. Spawner-isolated Mortality Virus (SMV) detection test development and refinement

Methods

Faecal Collection. Faecal samples were boiled immediately after collection in TE buffer (20 mM Tris-HCL, 10 mM EDTA at pH 8.0; Sambrook *et al.* 1990) as laboratory studies had shown that nucleases present in the faeces quickly destroyed any viral DNA present. The samples were then stored at –20°C until further processing.

DNA Extraction from Faeces. The method used to extract DNA required the exclusion of melanin from the extract which inhibits PCR (Giambernardi *et al.* 1998; Price *et al.* 1999). Samples were vortexed to disrupt the faecal strings and digested with 200ug/ml proteinase K (PK) and triton X-100 for 2hrs at 56°C. The DNA was then selectively attached to silica and proteins denatured with a chaotropic agent (NaI) (Progen Industries, Bandpure™). The nucleic acid extract was washed and eluted as per manufacturers instructions. The DNA was resuspended in a final volume of 30ul of 10mM Tris-HCl pH 8.0. Five microlitres of the extract was used in a 25ul PCR reaction.

Hybridisation. The *in situ* hybridisation method used was based on that described by (Rolighed and Lindeberg, 1996) with some modifications. Briefly, tissue sections were deparaffinised through xylene, washed several times in ethanol and rehydrated in distilled water and finally, equilibrated in TNE (50 mM Tris-HCl pH 7.4, 10 mM NaCl, 1 mM EDTA) buffer. Tissues were then made permeable to probe by digestion with proteinase K 100 µg/ml at 37-56°C for 10 min. Digestion was stopped by addition of glycine to a final concentration of 0.2% weight/ volume (w/v). Tissues were then stabilised in 0.4% paraformaldehyde for 5 min at 4°C. This was followed by two washes in double distilled water for 5 min after which tissues were air dried for 5 min. Probe was then applied at a concentration of 1 ng/ µl in hybridisation buffer (50% formamide, 5% dextran sulphate, 0.5 mg/ ml sperm DNA, Denhardt's solution, 4x SSC (0.15 M NaCl, 0.015 M sodium citrate at pH 7.0) a coverslip applied, then tissue and probe DNA denatured simultaneously at 95°C for 8 min. Slides were immediately quenched on ice for 1min and hybridised at 37°C overnight. Nonspecifically bound probe was removed by washing in 2x SSC twice for 5 min at room temperature and once in 0.1x SSC at 37°C. Detection of bound probe was carried out as per the protocol described by (Durand *et al.* 1996) with colour development carried out overnight. Tissues were counterstained with 0.5% aqueous Bismarck Brown for 30 sec and mounted with Crestia (Scientific Supplies) and Depex (Gurr).

Results and Discussion

5.1 Polymerase Chain Reaction (PCR):

PCR primers (SMV260 primers) were designed from sequence data available from initial sequencing of first several hundred base pairs of the viral fragment (BE6). They produced a 260bp amplicon. Efforts were directed towards optimising the PCR for use in faecal samples, as initial histopathological data indicated that spawner-isolated mortality virus was enteric in natural infection. Using faecal samples also provides a non-invasive method of diagnosis, allowing the monitoring of valuable animals like spawners. It also allows a tracing of disease

progression or detection of intermittent shedders that would not be otherwise possible where animals need to be sacrificed. The use of faecal samples presented several challenges in terms of PCR detection. Faeces contain several well known inhibitors of the polymerase enzyme used for the PCR (Gelfand *et al*, 1989). They also contain enzymes that destroy exposed viral DNA. So efforts needed to be directed towards optimising DNA yield and thus the chance of detecting low numbers of virus while eliminating as much of the inhibitors and destructive enzymes as possible. We have achieved a detection limit of approximately 10^2 gene copies of viral DNA using primarily PCR on faecal samples spiked with the cloned viral DNA.

The spawner-isolated mortality virus has not been isolated from prawns reliably since early 1996. So testing of the PCR detection assay has been based on the spiking of samples with cloned viral DNA BE6. Faecal samples were collected from commercial hatcheries and tested with the SMV260 primer set.

However, the SMV260 primer set also has some shortcomings. The primers were designed based on the limited sequence data available initially. The initial sequence of the viral insert is quite AT rich. This means that the SMV260 primers need quite low temperatures to anneal effectively to target DNA. Such conditions make the PCR more prone to the production of spurious bands especially where the amplicon size is small as is the case with the SMV260 PCR. This problem was evident in some of the samples examined from the above prawn hatchery which confounded the results, as multiple bands were present along with bands of the appropriate size of 260bp. With the completion of the sequencing of the viral DNA insert BE6, another set of primers was designed. These have a higher percentage of GC nucleotides and thus can tolerate higher temperatures, increasing the specificity of the primer set. The PCR conditions for this primer set, SMV200, were optimised for sensitivity and specificity. A combination of two sets of PCR primers (SMV260, SMV200) and a DIG-labelled dot blot confirmation test (SMV150) for one of the amplicons (SMV200) was used. The SMV200 with the internal dot blot (SMV150) was more robust and this test predominated with time. This test would detect about 100 copies of the genome of SMV.

5.2 *In situ* Hybridisation (ISH):

In situ hybridisation has been refined in terms of specificity and sensitivity of the probe. The probe which is the entire BE6 2000bp viral DNA insert described above has been used to examine tissue sections positive for MBV, HPV, IHHNV and Mycoplasma. None of the inclusions in these slides cross-reacted with the SMV probe. The probe also reacts consistently with presumed SMV cases from bioassays conducted with infective material by Jang Loo at James Cook University in 1995. Archival tissue samples have also been examined from farmed prawns collected in 1997, 1996, 1993 and 1992; and from prawns from Australian Institute of Marine Science (AIMS) collected in 1997 and 1992; bioassay animals at James Cook University from 1996 and prawns from a 1997 epizootic at Commonwealth Science and Industry Research Organisation (CSIRO) in Brisbane. The *in-situ* hybridisation test was then further refined by using restriction enzymes to cut the 2kb insert-containing plasmid into a 400 base pair fragment that had higher penetration into tissue sections and had less chance of nonspecific binding. The 400 pb fragment was gel purified and labelled with DIG dUTP using random priming. This was used on all subsequent sections including the crayfish. Positive specimens have been detected in several of these samples (Table 1) with the overall prevalence at 58% from 216 prawns.

Table 1: Archival tissue from penaeid prawns positive by *in situ* hybridisation with SMV400 probe

Year	Source	Positives	Prevalence %
1991	IHHNV epizootic[1]	0/9	0
1992	Farm epizootic	22/41	54
1993	Farm epizootic	8/18	44
1995	Spawners	6/8	75
1995	Bioassay of Spawners	8/9	89
1997	Farm mortalities	34/60	57
1998	Bioassay from farms	20/25	80
1998	farm mortalities	22/38	58
1998	Philippines	5/8	63
Totals		125/216	58

[1]Owens *et al* 1992

Experimental production of infected material: In one trial prawn material was produced from transmission trials specifically for in-situ hybridisation (ISH) and electron microscopy (EM). The aim was to evaluate the probe but also to gain a better understanding of the virus(es) involved in MCMS. Ultrafiltrates were prepared from prawns collected at a farm epizootic (early 1997) and from 3rd passage of infected material of a farm epizootic in 1995. Self-controls were run at the same time. Untreated prawns were sampled at the beginning of the trial for ISH and EM. Sick and moribund prawns were collected and processed through the trial as detected. Moribund prawns appeared at day 7 and the remaining prawns were sampled on day 11 (1997 material), day 13 (3rd passage material) and day 20 for the self-controls (only a 50% survival). Again, the experimental prawns already appeared to have subclinical infections.

Electron microscopy of prawns receiving the 1997 epizootic material revealed the presence of okavirus (GAV) in all necrotic tissues, including antennal gland cells which had a diffuse to extensive necrosis only present in prawns experimentally inoculated with cell-free extract. No other virus-like particles were observed in any tissues. This trial does lead to the conclusion that SMV virus, originally isolated from 1995 MCMS outbreaks, is not always necessary for MCMS epizootics or transmission of infection.

Experimental prawns in the mode of transmission trial were collected for histology (then ISH) and electron microscopy. Five prawns were sampled before the trial and prawns surviving at the end of the trial were fixed for EM and/or histology. Again all samples were negative with the probe. The okavirus (GAV) was detected at EM in tissues of some of the prawns.

6. Source of virus

6.1 Viral Taxonomy

Sequencing of the ~2kb clone of SMV has allowed it to be compared to other viruses. Analysis of the use of gene promoters, start and stop signals and processing signals suggest that SMV is most closely related to the genus *Dependovirus* of the family *Parvoviridae* (Table 2). This is further supported by the analysis of the frequency of codon usage for the amino acids arginine, luecine and serine based on the open reading frames predicted from the above start and stop signals (Table 3). The use of codons for amino acids in SMV is most similar to Adeno-Associated Virus (*Dependovirus*) but no significant difference in codon usage is apparent (Table 3). However, electron micrographs of primary cell cultures derived from penaeid prawns that have been infected with SMV PCR-positive material did not show any other virions except SMV (L. West and R. Webb, personal communication). This suggests SMV is an autonomous virus rather than a strict *Dependovirus* that needs helper viruses to replicate. The *in-situ* hybridisation data suggests the virus is most often in non-cytopathic nuclei and cells which does suggest it may be integrated into the prawn genome and only rarely produces full virions. The analysis of the arrangement of the viral genes supports this hypothesis as the main capsid gene occurs after the "strong stop codon" (Table 2) suggesting limited translation of this gene under normal conditions is likely.

Collectively, this data indicates that SMV is probably related to both the autonomous genus *Parvovirus* and the genus *Dependovirus* with closest affinities to the genus *Dependovirus.*

The ~2kb shows no significant nucleotide homology with the overseas strain of the prawn parvovirus, Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) which has just been listed on Genbank.

Table 2. The sequence homology for start promoters and other codons used by the family *Parvoviridae* to spawner-isolated mortality virus. Comparative data from Rhode and Iversen (1990). AAV Adeno-Associated Virus; B19 Human Parvovirus; BPV Bovine Parvovirus; CPV Canine Parvovirus; FPV Feline Panleucopaenia Virus; GmDNV *Galleria mellonella* Densovirus; H1 Rodent Parvovirus; MVM Minute Virus of Mice; SMV Spawner-isolated Mortality Virus.

Promoter and Sequence	**Parvovirus**	**Densovirus**	**Dependovirus**	**Spawner-isolated Mortality Virus**
P4, TATAA	H1,BPV			
P4, TATAT	MVM			
P4, TATATA	B19	GmDNV		
P4, TATTTA			AAV	SMV
P13, TATAAT	BPV			
P19, TATTTA			AAV	SMV
P38, TATAAA	H1, CPV,B19			
P38, TATTAA	BPV			
P38, TGTAAA	FPV			
P40/45, TATAA	B19, MVM		AAV	SMV
Poly A AATAAA	FPV, B19, BPV, MVM, H1		AAV	SMV
Strong Stop GGAGATACA T	MVM, H1			SMV GTAGATACA T
Replicating Nick Site CTATTC	MVM			SMV

Table 3: Mean distance between various parvoviruses and spawner-isolated mortality virus SMV calculated by codon usage for arginine, luecine and serine. Comparative data from Iversen and Rhode (1990).

Virus	Adeno-Associated Virus AAV	Human Parvovirus B19	Rodent Parvovirus H1	Minute Virus of Mice MVM1
Mean Distance %	**10.71**	**15.22**	**13.49**	**16.68**
SE Mean	2.68	3.36	2.24	3.69
Sig	P>0.05	P>0.05	P>0.05	P>0.05

6.2 Archival prawn tissue testing

The earliest prawns detected by in situ hybridisation (ISH) to be infected with SMV were from a farm experiencing unexplained mortalities in 1992. Subsequently in 1993, spawner

prawns from the Australian Institute of Marine Science (Fraser and Owens 1995) were ISH-positive as were prawns from farms in 1995, 1996 and 1997. However, prawns from many epizootics from 1996 onwards were negative to SMV suggesting the dominance of GAV in these samples.

Similarly, the first crayfish farm that was positive by in situ hybridisation was also in 1992 (Table 4). The same farm was positive in 1996 and many farms were found positive in 1998/9 when surveyed.

Table 4: Archival tissue from freshwater crayfish, *Cherax quadricarinatus,* positive by *in situ* hybridisation with SMV400 probe.

Year	Farm	Positives	Prevalence %
1992	1	13/44	30
1992	2	0/10	0
1992	3	0/10	0
1992	4	0/10	0
1992	5	0/10	0
	Subtotal	13/84	15
1996	1	8/10	80
1998/99	15 farms	12/15 farms; 49/121	40

The data from both the prawns and crayfish suggest that SMV has been in farms in northern Queensland since 1992. Sufficient samples before this date have not been examined. Of note is the fact that the 1991 epizootic ascribed to Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) (Owens et al 1992) (Table 1) showed no reactivity to SMV. This suggests the viruses were different and SMV was not involved in the 1991 mortalities.

Another question to be answered regarding the source of the virus was the possibility that SMV was a mutation of Hepatopancreatic Parvovirus (HPV) which is known to occur in Australian *Penaeus merguiensis*. Both viruses are parvoviruses with the same tissue tropism and HPV does not appear to express as the classic pathognomonic lesion in Australian *Penaeus monodon.* It was possible that SMV was a non-classical expression of HPV. Examination of 226 *P. monodon* for HPV using an *in situ* gene probe failed to find a single case of HPV. Twenty-two cases were selected, as they were positive for SMV (listed in Table 1) but they showed no staining for HPV. This suggests that SMV is not a mutation of HPV but a distinct virus. This information has been submitted for publication Kahn *et al* (submitted).

In summary, SMV has been in prawn and crayfish farms since 1992 and it was associated with ill thrift since that time. Its existence prior to 1992 has not been investigated. SMV appears to be a unique virus and not a mutation of a previously discovered virus. There is a major route of infection from spawners to progeny that has been identified (see section 9).

6.3 Archival prawn tissue testing using commercial *in situ* hybrization tests for exotic virus.

From the time a viral aetiology for MCMS was demonstrated, it has been assumed that the virus(es) involved were endemic and not exotic (introduced) viruses. This part of the project aimed to produce evidence that MCMS was not caused by, nor the same as diseases reported from other countries culturing prawns. At the same time known endemic viruses (MBV and HPV) in Australia were tested to evaluate the value of the ISH detection tests from USA.

Methods.

Commercially available *in situ* hybridisation (ISH) kits produced by DiagXotics for detection of prawn viruses were imported. The ShrimProbe® kits for White Spot Virus (WSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Taura Syndrome Virus (TSV), Yellowhead Virus (YHV), Monodon Baculovirus (MBV), *Baculovirus penaei* (BP) and Hepatopancreatic Parvovirus (HPV) were used on tissue sections from selected case material in storage at the Oonoonba Veterinary Laboratory.

The ISH followed exactly the methods described in the kit instructions for each ISH. A control tissue section (supplied with the kit) was probed at the same time as the selected tissue section. Briefly the method was that the paraffin sections on glass slides were heated, washed and then treated with Proteinase K. The slide was then washed and treated with formalin solution, then the hybridisation solution was added and the slide incubated for the specified time. After the incubation the slide was washed then incubated with antiDIG/AP conjugate. After this incubation the slide was washed then incubated with the development solution. Finally the slide was washed and counter stained in Bismarck Brown then mounted and examined microscopically. Where present the typical tissue lesion was specifically examined for a positive probe reaction.

Archived, parafin-embedded tissues from submissions for diagnosis of disease in juvenile *P. monodon* (from earthen production ponds) and health testing of batches of post-larval *P. monodon* were retrieved. All the cases retrieved from storage were submitted from prawn farms and hatcheries north of Mackay on the east coast of Queensland. The selected case material (tissues) were:

1. Lesions examples: Six blocks of prawn tissue were selected to represent the range of common tissue lesions seen in diseased juvenile prawns in north Queensland. These included the common viral infections (MBV, HPV and LOV); characteristic tissue lesions only seen in juvenile prawns from ponds with MCMS (intranuclear inclusions in anterior caeca and focal nerve necrosis); and the only example seen in Queensland of 'white spot' epithelial necrosis in juvenile farmed prawns. These 'lesion example' tissues were tested with all the above ISH kits.
2. MBV examples: Histological blocks from cases where MBV was diagnosed in post-larvae (8 different cases) and in juveniles from ponds (3 different cases) were selected for testing with the MBV and BP ISH detection kits. Blocks from two cases of healthy post-larvae were included as control tissues.
3. HPV examples: Histological blocks from cases where HPV or suspect HPV was diagnosed in post-larvae (5 different cases) were selected for testing with the HPV ISH detection kit. Five cases of healthy post-larvae were included in this group as control tissues.

4. Diseased juvenile prawn examples: Histological blocks from cases where dead and sick juvenile prawns, including blocks from the MCMS index cases, were selected to determine if any of the tissue changes present were associated with exotic viruses. Consequently tissues from 14 different farms, and 36 diferent disease outbreaks, from Sarina to Cooktown on the northern east coast of Queensland were tested with all the above ISH detection tests.

Results and Discussion.

Six of the sections with MBV lesions returned a positive to the DiagXotics ISH test, only one of the infected juvenile prawns. None of the suspect HPV infections returned a positive to the ISH test. This could be explained by some of the findings of Kahn *et al* (submitted) where modification of the DiagXotics ISH procedure was required to get consistent positive results from HPV positive control sections prepared from wild-caught *Penaeus merguiensis*.

No positive reactions were observed in the tissues from the lesions examples or any of the tissue sections from the diseased juvenile prawn examples. This was not an epidemiologically designed survey and the ISH has a much lower sensitivity to viruses as compared to a PCR detection test which are recognised as the 'gold standard' for surveillance testing. As a consequence this information cannot fulfil the requirements for international recognition of freedom-from the exotic viruses. But as the selection of prawn tissues was biased towards diseased prawns, these results strongly indicate that the disease problems present in the Queensland prawn industry were not associated with any of the exotic viruses detected by the ISH tests used here.

7. Live virus transmission trials

7.1 Live virus transmission trials – preliminary research

Aim

1. In the absence of specific histopathology or, at the beginning of the project, the absence of specific detection tests it was necessary to establish a bioassay system to detect the presence of MCMS agents in prawn carcases or tissues.
2. In the absence of *in vitro* live virus culture systems, a standard bioassay system was also required to evaluate the effect of physical and chemical treatments on MCMS virus(es).

Methods

Preparation of ultrafiltrate

A standard method was used to prepare an ultrafiltrate from MCMS infected prawns. The infected material used was either clinical material collected from MCMS epizootics or experimental, mass transmission trials specifically done to amplify the amount of infected material available for use in the project. Storage of infected prawn tissues was at -20°C for periods <8 weeks or at -80°C for long-term storage.

The prawn carcases were freeze-thawed three times, the abdomen removed and the thoracic cuticle dissected free from underlying soft tissues. This was done at 4°C and all subsequent processing was at 4°C. The thoracic tissue was placed in 4x volume of 2PBS and 0.5mM 4-hexylresorsinol and homogenised in a Sorvall homogeniser. The suspension was clarified by centrifugation at 4°C, 10,000g for 30 minutes and the supernatant filtered through a 0.45µm disposable sterile filter. The product from this process is termed ultrafiltrate as only infective particles smaller than bacteria would be present. A drop of the ultrafiltrate was inoculated onto Marine Agar and Blood Agar then incubated at 26°C for 24 hours to confirm the absence of bacteria. Ultrafiltrate was usually prepared just before it was required, if stored for any time this was at -80°C.

Standard mortality curve

Prawn carcases collected from three farms with MCMS outbreaks were used by Dr Paul Muir (seconded from James Cook University to the Queensland Department of Primary Industries) to originally demonstrate the transmission of a filterable agent and thus a virus aetiology for MCMS. The ultrafiltrate was prepared from carcases from each farm as above except that 4-hexylresorcinol was not used to inhibit melanisation.

All prawns used in these and subsequent trials were commercial breed and reared *Penaeus monodon*. The size of the prawns varied from batch to batch depending on availability, but prawns less than 10 grams were preferred due to their manageable size. They were collected from ponds on farms with no recent history of prawn mortalities and where it was reported they had normal feeding.

Four 60 litre glass aquaria with constant aeration, a corner filter with filter wool were set up with 10 experimental prawns in each. Water was exchanged as necessary to maintain water quality, this usually meant that a 60-80% exchange occurred 3 times a week. All seawater

used at the Oonoonba Veterinary Laboratory comes from Cleveland Bay off Townsville. The water is stored in holding tanks for more than a week where sediment settlement occurs. No other treatment of the water, other than pre-heating if necessary, was done prior to transferring to aquaria. Air temperature (and thus water) was maintained at 28±2°C. The prawns were feed a commercial pellet food *ad lib*, but not to excess. The prawns in each aquarium were injected in the abdomen with 0.05ml of ultrafiltrate prepared from the carcases from a single farm. The fourth aquarium was a negative control where the 10 prawns were injected with 0.05ml of 2PBS.

Effect of water temperature

Early in the studies on MCMS a small trial accidentally demonstrated the effect of temperature on disease development. Two groups of 12 prawns in 60 litre aquaria were maintained as described above. One group received 0.05ml ultrafiltrate prepared from prawn carcases collected at one of the farm epizootics, the other 0.05ml 2PBS. The ambient air (and thus water) temperatures were 20-21°C at the start of the trial. As no mortalities were observed within the time after infection as expected, water was heated to 27.5°C. Mortalities immediately followed.

Effect of passaging on pathogenicity of MCMS agent(s)

To facilitate repeated transmission trials it was necessary to produce large numbers of infected prawn carcases. As there could be an attenuation of virulence of the MCMS virus(es), a comparison of the mortality curves produced with ultrafiltrates made from second and third passage prawn carcases was done. Second passage material is where the moribund prawns from trials where experimental prawns were injected with ultrafiltrate prepared from prawn carcases collected at a farm epizootic. A third passage is where the ultrafiltrate was prepared from moribund prawns collected from a second passage transmission trial. The original clinical material came from one of the farms shown in the standard mortality curve and used in the above temperature effect trial.

The preparation of ultrafiltrates and care of experimental prawns was as described above. The second passage mortality curve was the mean of two groups of prawns which totalled 81 prawns on Day 0. The third passage mortality curve was from a single group of 50 prawns stocked at Day 0. Maintenace essentially followed that described for the 'standard mortality curve' trial, although 1,000 litre tanks were used to hold the experimental prawns. Air temperature (and thus water) was maintained at 28±2°C.

Results and Discussion

Standard mortality curve

The pattern of mortalities seen after injection of ultrafiltrate prepared from clinically diseased prawn carcases had a fairly consistent pattern (Figure 2). Mortalities would begin 9 to 12 days after parental injection and then rapidly declined to 19 to 21 days, often with an occasional mortality observed until 25 days. This established the period any experimental transmission study would need to run. For this project a one month period became the standard for transmission trials.

Interestingly the delay between infection and the rapid mortalities expected in a virulent viral disease in prawns was quite long. In studies with the major viral disease overseas, White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV), experimental transmission results indicated mortalities as early as 2 days post infection (Lu *et al* 1994; Chou *et al* 1995), with 100% mortalities by 5 to 7 days. This preliminary work indicated a viral agent was involved but that MCMS virus(es) was a new type of viral disease in prawns.

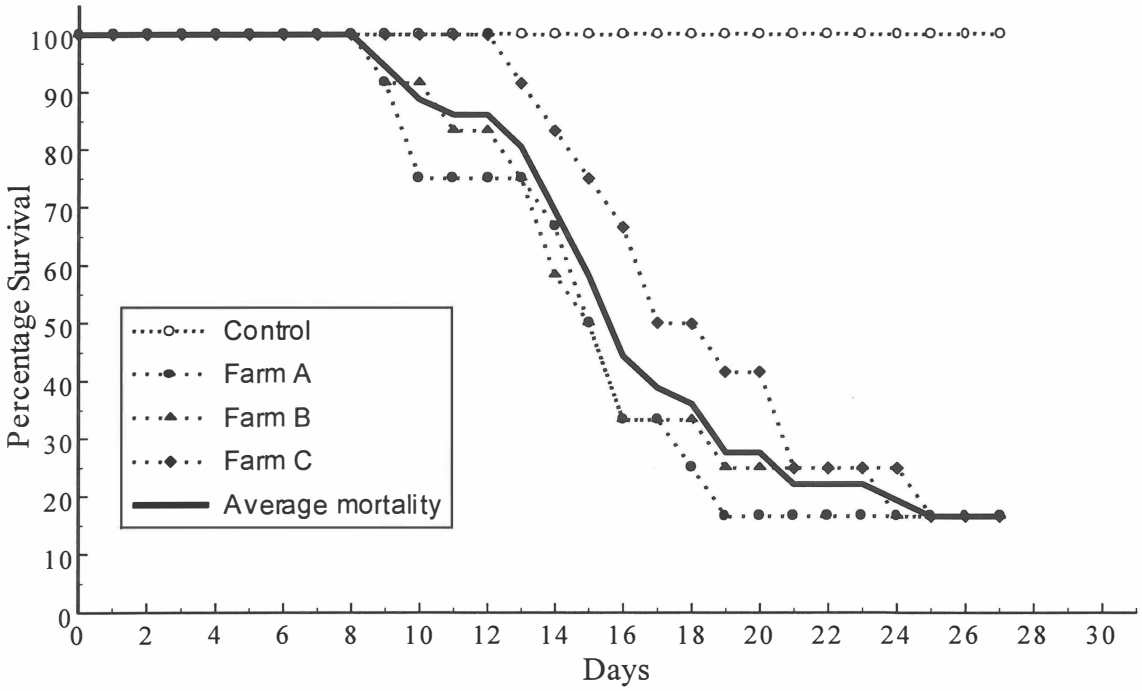

Figure 2: Mortality curves (at 28°C) produced by injected ultrafiltrate prepared from moribund prawn carcases collected from three different farm epizootics. Each curve for Farms A, B and C are records from one tank of experimental prawns.

Effect of water temperature

While this trial was only done once, it did demonstrated the effect of water temperature on the expression of MCMS (Figure 3). As soon as the water temperature was increased to 27.5°C mortalities began. This indicates the injection of ultrafiltrate did result in infection but the infection was not active. The higher temperature allowed the MCMS virus(es) to replicate and cause clinical disease and mortality. This reflected the clinical appearance of MCMS in 1996 where on-farm epizootics ceased during the winter and we saw epizootics begin to occur again in the spring once water temperatures increased again.

Flegel *et al* (1997) describe how, in Thailand, the presence of WSSV or YHV in a cultured prawn population does not mean prawns cannot be grown to market size. They indicate that a sudden change in pH or drop in water dissolved oxygen can 'trigger' an outbreak of yellow head disease. The role of stress in initiating clinical prawn virus diseases was highlighted by Chen *et al* (1989) as an important factor in the collapse of Taiwan prawn aquaculture. In China it was observed that WSSV disease spread more rapidly when water temperatures were greater than 25°C (Zhan *et al* 1998). Even the daily removal of a pereiopod was enough to cause clinical white spot disease in prawns carrying WSSV (Peng *et al*, 1998). Whether the onset of mortalities in this trial was a stress effect due to the sudden increase in temperature

or that a more complex host-pathogen interaction is involved is unknown, but the finding is a useful when interpreting disease outbreaks on prawn farms.

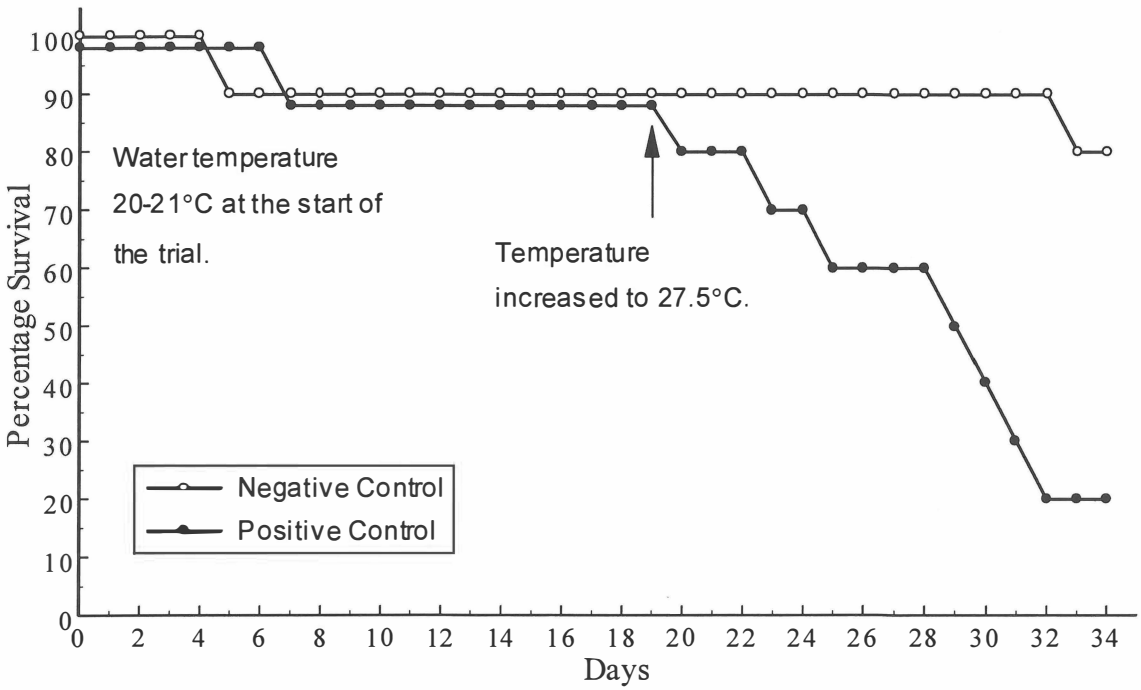

Figure 3: The delay in the mortality of prawns injected with ultrafiltrate prepared from prawn carcases collected at a farm epizootic due to a low water temperature.

Following this result all subsequent trials, even those done during the winter, were done with water temperatures held at 28±2°C. This was to ensure successful virulent infections would be established. This was achieved at OVL in the Isolation Building with initially radiant heaters in each experimental room and then with reverse cycle air conditioning.

Effect of passaging on pathogenicity of MCMS agent(s)

As can be seen in Figure 4, there was no reduction in virulence of the MCMS agent(s) on passage through live experimental prawns. While the mortality curves seen showed mortalities begin soon after injection of ultrafiltrate, the rapid increase in mortalities matched that seen in the standard mortality curve. That is to say mortalities increased around Day 9/10 and continued until Day 20/21, with occasional mortalities observed until the end of the experiment. This matched closely the standard curve produced when using clinical material from farm epizootic.

The conclusion was that amplification of MCMS infected material through experimental, mass transmission trials and its use in subsequent transmission trials was scientifically valid. This is similar to YHV where passage of upto seven times had no effect on virulence of the virus (Boonyaratpalin *et al* 1993).

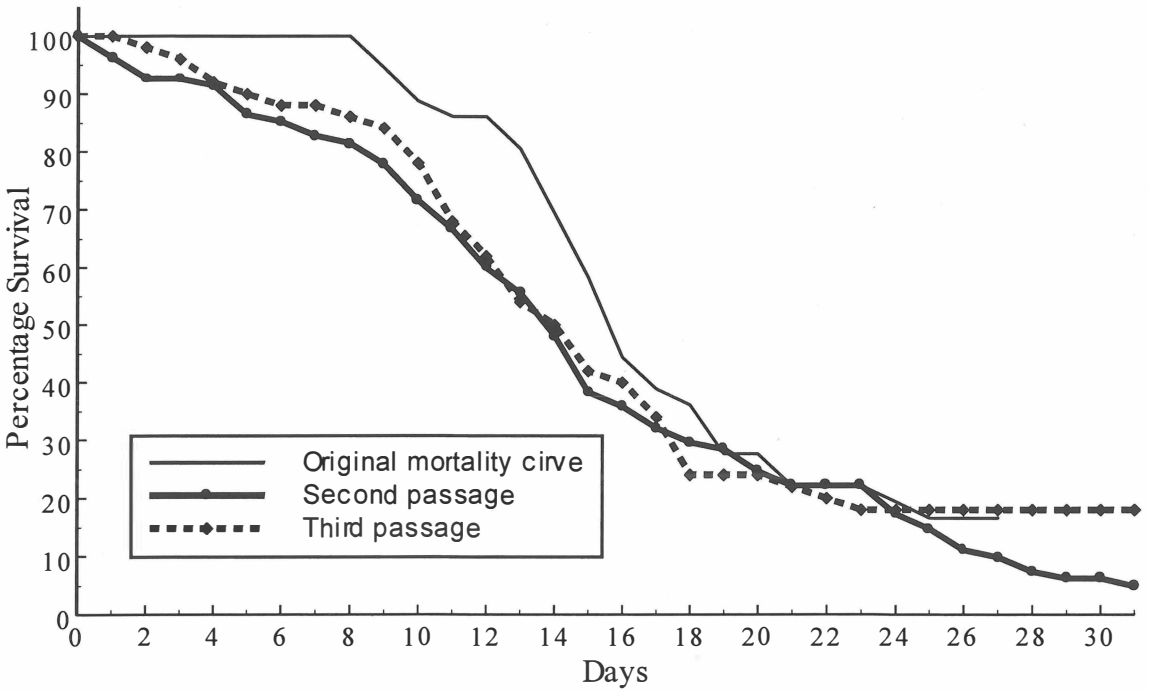

Figure 4: The effect on the mortality curve (at 28°C) produced by ultrafiltrates produced from prawn carcases from clinical material, second and third passage material.

7.2 Purification of MCMS virus(es)

This project had a focus on SMV, although it is known that several viruses were associated with MCMS epizootics. It was proposed that we use only purified virus suspensions of SMV to do transmission trials. This is the best approach to the study of viruses. In the absence of crustacean cell lines the purified virus suspensions would have to be produced from infected prawn material.

Methods.

Purification: In the case of purification trials the standard procedure involved thawing infected prawn carcases, dissecting all soft tissues from the thorax and placing in a small amount of TNE (100 mM Tris, NaCl and EDTA) and 4-hexylresorsinol (HR). The tissue was homogenised, the homogenised tissue suspension was clarified, then centrifuged at 4°C, 40,000rpm for 3 hours and the resulting pellet resuspended in a small volume of TNE. This was layered on a 1.2 to 1.5 g/ml continuous CsCl gradient and centrifuged overnight. The gradient was then examined for bands. The buoyant density of the band determined for index measurement and the bands examined by transmission electron microscopy.

Variations trialed:
1. Homogenisation: Sorvall homogeniser, ultraturrax or ultrasonication.
2. Clarification: One centrifuge step or three centrifuge steps with the final step at 12,000rpm for 2 hours.
3. Gradient centrifugation: Caesium chloride (CsCl) (20 to 45% linear gradient), sucrose (15 to 50% linear gradient) or urografin (15 to 47% linear gradient) with centrifugation at 38,000rpm for 17 hours.

Infected tissue source: Three different sources of infected material were used (all at the same time in each trial). These originated from an amplification trial, clinical material collected at an epizootic and material collected from a batch of experimental prawns which developed an MCMS epizootic on holding in OVL tanks.

Results and Discussion.

The first trials using all the homogenisation methods, the one step centrifugation for clarification and the CsCl gradient resulted in no bands, except expected one for haemocyanin (the oxygen carrying molecule), being visualised.

With the three step clarification a 1.36 g/ml band was produced from amplification trial material and the clinical material. Examination of the band under TEM failed to reveal virus particles. Further 1.36g/ml bands were produced, pooled, and concentrated. Again TEM failed to detect and virus.

Following the methods originally used by JCU for the isolation of the SMV virus, the 1.4g/ml band containing the parvo-like (densovirus) particles obtained by JCU on several occasions has not been evident in our preparations.

In the trials using different gradient centrifugation, tissues were dissected in TNE and 4-HR with 3% SDS, clarified at 5,000 rpm for 30 min and pelleted at 50,000g for 4 hours. Pellets were resuspended and layered onto the sucrose or urografin gradients and centrifuged at 25,000rpm for 1 hour. Six 2ml fractions were taken from each gradient type, pelleted and resuspended. Subsamples from each fraction were viewed by TEM and were used to inject into 5 experimental prawns.

Virions were observed in urografin fractions taken from about a third the way down the gradient. Icosahedral particles of 25nm were present, but also detected were phage particle with heads about the same size. When this fraction was injected into prawns, no mortalities were seen.

It was some what surprising to find we could not purify any virus(es) from clearly diseased prawn tissues. Research reports from other countries indicate that both YHV and WSSV could be purified through step-wise centrifugation culminating with a sucrose or urografin gradient (Kimura *et al* 1995; Nadala *et al* 1997; Wongteerasupuya *et al* 1995). GAV, the other main virus associated with MCMS, has also not been purified in Australia (P. Walker, pers. comm.). It is not clear why the purification attempts failed, the techniques used were appropriate for other prawn viruses.

After these trials it was decided to abandon attempts to use purified virus suspensions for transmission trials. All transmission trials in the project used the ultrafiltrate produced as described earlier. In doing this we had to accept that more than one virus could be present in the ultrafiltrate and as a consequence we refer to transmission of MCMS and not SMV.

7.3 The infectious dose of MCMS virus(es)

Initial research by project staff and others suggested that the different viruses associated with MCMS would produce different mortality curves. Another view was that any differences in the on-set of mortalities and the steepness of the mortality curve was due to different infectious doses. Consequently, there was a need to do a dose-response study using dilutions of ultrafiltrate (and thus virus(es)). Determining a minimum infectious dose was also needed. This would help evaluate risks of transmission on farms in outbreaks and plan management strategies – how many prawns would need to be transferred to transfer disease. A minimum infectious dose would also be useful in evaluating disinfection effects.

Method.

Two successful trials were completed where negative and self- control prawn survivals were close to or greater than 80%.

Experimental prawns were obtained from prawn farm ponds with no recent history of ill health. In the first trial, the prawns had a mean weight of 12.3 grams (range 9.2 to 13.5 gram). In the second trial, the prawns had a mean weight of 9.8 grams (range 6.7 to 11.2 gram). The prawns were maintained in 120 litre aquaria with continuous aeration and two internal corner filters containing coral rubble and filter wool. Water temperatures were held at 28 $\pm$ 2°C. Water quality was maintained by three 80% water exchanges per week and the filters were cleaned once a week. The prawns were fed a commercial pellet food *ad lib* but not to excess. In the first trial 6 prawns and in the second trial 10 prawns were used in each aquaria.

The experimental design was the same for both trials. Each treatment was duplicated. There were three control treatments; untreated control, self control (injected with ultrafiltrate prepared from a sub-sample of the group of experimental prawns) and a positive control or the 10^0 dilution (injected with ultrafiltrate prepared from prawn carcases collected from a farm epizootic). Six dilution treatments were used. These were prepared as a 1:100 dilution series (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of the positive control ultrafiltrate. 0.05ml of undiluted or diluted ultrafiltrate was injected into the abdomen of the experimental prawns on day 0. The trials were run for 35 days.

Results and discussion.

One of the major constraints in the project was the difficulty in obtaining healthy *P. monodon* juveniles from farms for experimental studies. While great care was taken in sourcing from farms and ponds where the farm reported good survivals, growth and active feeding, it became apparent that these populations could express clinical MCMS following stress through capture, transport and holding at the laboratory. Groups which showed poor survival on holding were not used. Despite this care we would often start a transmission trial only to find control survivals were poor making any information on survivals in treatments invalid. The absence of detection tests for viruses or other assessment to determine disease status meant that this happened quite often. A self-control, experimental control was introduced into all transmission trials using *P. monodon* as experimental prawns. This was an attempt to more accurately assess the health status of the experimental prawns to ensure transmission trial results could be accepted. It was clear the stress of transport, handling, stocking into aquaria and injection was sufficient to trigger clinical disease in apparently healthy *P.*

monodon. In a way, the preparation of an ultrafiltrate from a sub-sample of the batch of juveniles to be used as experimental prawns was a way to assess viral infection intensity. If the prawns carried a high intensity infection, the self-controls had poor survival. If the prawns had only a light infection, and self-controls had a good survival – around 80% or better, we assumed the virus(es) we injected would be the dominant factor controlling survival. It must be noted that the measurement of survival in transmission trials does not assess infection *per se*, but a lethal, active infection. The results presented here were the sixth and seventh attempt to determine the effect of different infectious doses.

The results from both trials were combined and the mean plots of the mortality curves is given in Figure 5.

The mortality curve produced by the lower dilutions of ultrafiltrate are similar to the control (un-diluted) ultrafiltrate. As expected the 10^0 dilution mortalities rapidly increased 9 days post-infection until levelling of at 19 days. This is very similar to the standard mortality curve. The 10^{-1}, 10^{-2} and 10^{-3} all had a similar rapid increase in mortalities, but the onset was delayed to 12-15 days. This would represent the period of incubation needed for initially lower virus(es) numbers to replicate. The 10^{-4} dilution had a longer incubation (18 days) and slightly shallower mortality curve, but one still close to the standard mortality curve.

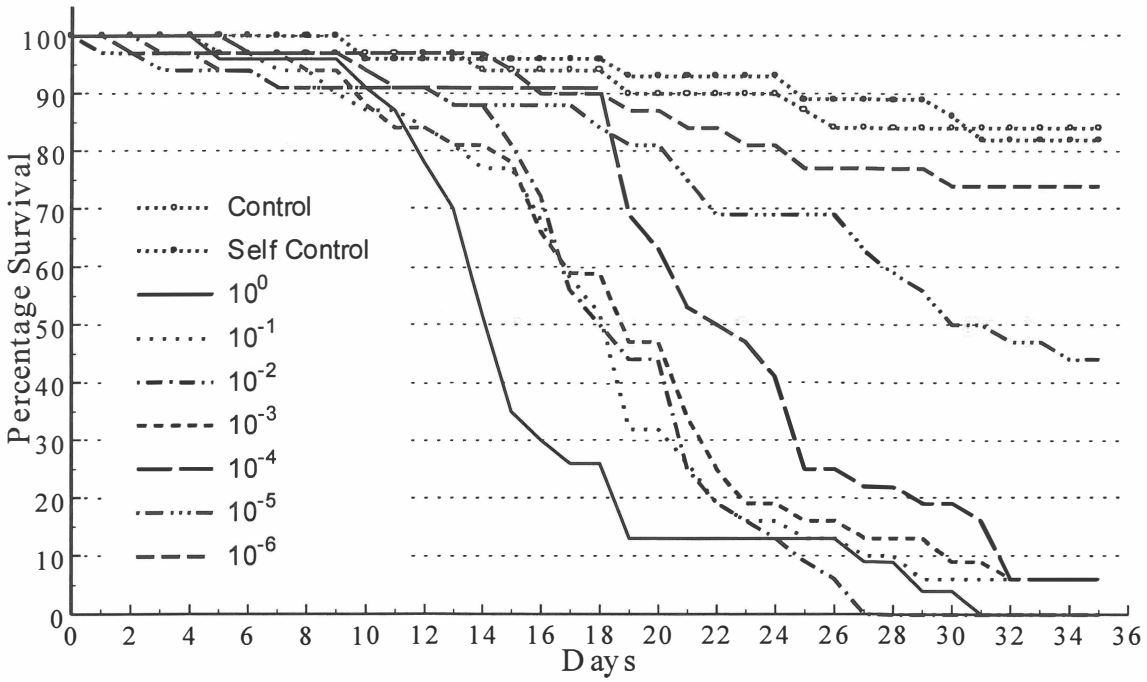

Figure 5: The effect of different concentrations of ultrafiltrate (infectious dose) on the survival of experimental prawns (mean data from two duplicated trials).

It was not until the 10^{-5} dilution that a different mortality curve was noted. The increasing mortalities began around 16 days but the curve was not as steep as the seen with the lower dilutions. The results suggest the 10^{-5} dilution is an ED_{50} (Effective Dose 50%) dose at 30 days post-infection. The interpretation of the 10^{-6} dilution is difficult as the results are not greatly different than the untreated and self control treatments. Similar to many of the 'good' transmission trials, both control treatments did have mortalities but as survivals at 30 days were greater than 80% it is considered the trials reported were valid. Due to the cannibalistic

nature of *P. monodon*, recently moulted prawns are eaten and killed by other prawns in the aquarium. Usually a carcase with no appendages is found, but occasionally an entire prawn carcase can be eaten overnight.

There is little information in scientific reports which describe the minimum infectious dose of different prawn viruses. Recently Wang and Chang (2000) reported a $2x10^{-4}$ dilution of purified YHV successfully caused a lethal infection, causing 86% mortalities in experimental prawns after 7 days. This more diluted inoculum resulted in a slight delay in mortalities similar to the more diluted ultrafiltrate treatments in these trials.

From the results it is possible that the 10^{-6} dilution did result in an infection to cause a chronic, lethal infection in the experimental prawns. A longer trial would be required to clearly demonstrate if 10^{-6} is the threshold (minimum) dose. In any event, considering the ultrafiltrate is produced as a 1:4 dilution of the original volume of tissue, a 0.05ml volume of a 2.5×10^{-6} dilution of infected tissue implies that a minimum infectious dose, by injection, is present in a very small amount of diseased prawn tissue.

These results lead us to select 10^{-1}, 10^{-3} and 10^{-6} dilutions of ultrafiltrate to evaluate virus destruction methods.

7.4 The route of infection of MCMS virus(es)

These trials were done to determine if MCMS could be transmitted by oral and water borne routes.

Method

Experimental prawns were obtained from a prawn farm pond with no recent history of ill health. The prawns had a mean weight of 12.3 grams (range 9.2 to 13.5 gram). The prawns were maintained in 120 litre aquaria with continuous aeration and two internal corner filters containing coral rubble and filter wool. Water temperatures were held at 28 $\pm$ 2ºC. Water quality was maintained by three 80% water exchanges per week and the filters were cleaned once a week. The prawns were fed a commercial pellet food *ad lib* but not to excess, except for the first three days in the oral challenge treatments.

There were three control treatments, each one duplicated; untreated control, self control (injected with ultrafiltrate prepared from a sub-sample of the group of experimental prawns) and a positive control (injected with ultrafiltrate prepared from prawn carcases collected from a farm epizootic). There were three replicates each of the oral and water borne challenge treatments. Each replicate of the water borne group shared common water with the corresponding replicate of the oral challenge group by way of a filtration system (to remove particulate matter) with the intake in the oral challenge tank and the outlet in the water borne challenge tank. The water borne challenge tank was elevated so that the excess water drained back to the oral tank via an overflow hose which was inserted through the water borne tank approximately 2 cm below its top. All replicates contained 10 prawns.

Infected material from the same farm epizootic as used to produce the positive control ultrafiltrate was used as the source of infected material for the oral challenge. Soft tissues were dissected from the cephalothorax and cut into 5mm cubes. Prawns in the oral challenge

treatments were fed approximately 5% biomass per day in two feeds over the first 3 days of the trial (days 0, 1 and 2). 0.05ml of each control ultrafiltrate was injected into the second abdominal segment of each prawn in these treatments. The trial was run for 96 days.

Results and discussion

As discussed previously, one of the major constraints in the project was the difficulty in obtaining healthy *P. monodon* juveniles from farms for experimental studies. The results presented here in fact are the third attempt to determine the route of infection of MCMS virus(es).

The results from the trial are given in Figure 6. The replicates of each treatment were averaged (controls 2x replicate, oral and water borne challenge treatments 3x replicate) and plotted.

The injection challenge (positive control) again follows closely the standard mortality curve. There was a rapid increase in mortalities beginning at 8 days post-infection until levelling off at 20 days. The untreated and self control survivals were at 80 or 75% on day 95. This was good enough to consider the results from the trial valid.

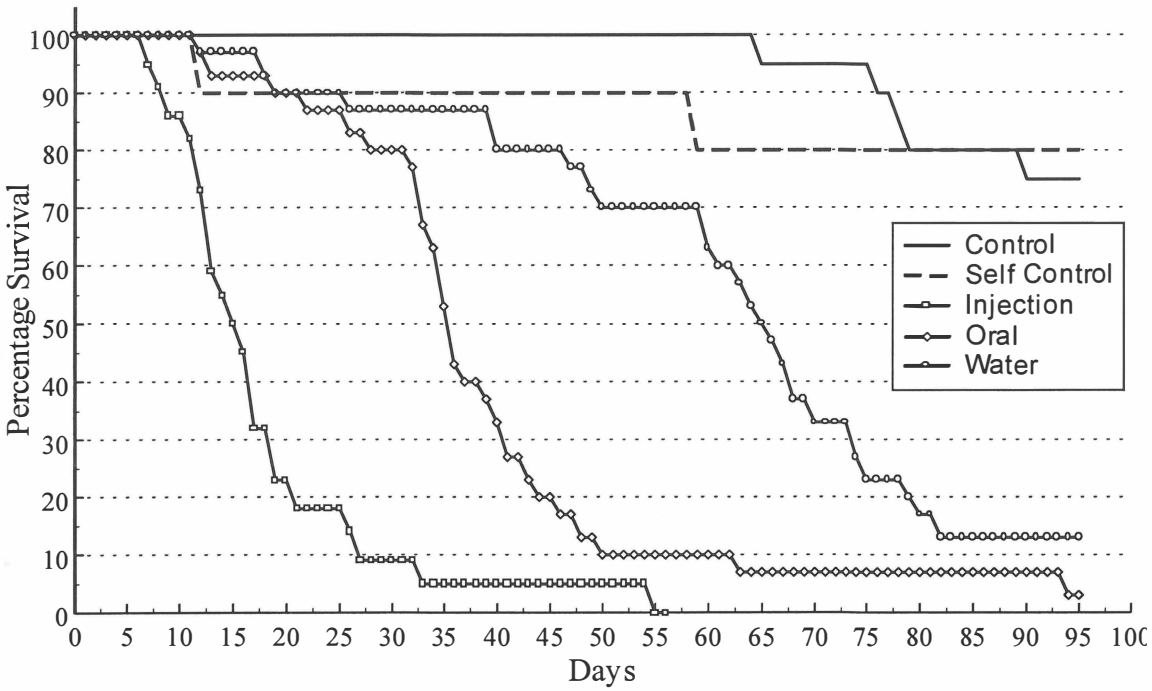

Figure 6: The effect of different routes of infection challenge by MCMS infected ultrafiltrate or tissues (mean data from replicated tanks).

The survival curve for the oral challenge was similar to the injection challenge but the onset of rapidly increasing mortalities was delayed until day 34. The results clearly demonstrate oral transmission of MCMS does occur. This is consistent with the clinical observations of epizootics on farms where it is considered transmission on a farm is the result of bird-mediated spread of diseased or dead prawns from a pond with MCMS to another pond. The infection then spreading within the pond with cannibalism of diseased prawns by healthy prawns. Even if an MCMS infection is introduced to a pond via the post-larvae, typically the

MCMS epizootic is not seen until mid-way through the crop ie., at 16 weeks or when the prawns are 12-16 grams, then it can be assumed the slow spread of the infection is the result of this oral transmission routes and the long incubation time (34 days). The absence of an early explosive crop failure was one the characteristics of MCMS (together with the absence of classic viral lesions) that made understanding the infectious viral nature of the disease syndrome difficult to identify when the disease initially started affecting northern Queensland prawn farms.

White Spot Syndrome Virus has been shown to be transmitted orally, but Sahul Hameed *et al* (2000) reported the first mortalities in *P. monodon* as early as three days after feeding infected prawn tissue. In similar trials assessing susceptibility of western hemisphere shrimp, feeding of WSSV infected tissue produced mortalities as early as 3 days in *P. vannamei*, while the first mortalities were seen by day 6 in *P. vannamei* and *P. aztecus* after feeding YHV infected material (Lightner *et al* 1998).

The water borne mortality curve clearly indicated that transmission via water is possible. The mortalities >20% did not occur until day 48 and the curve tended to be shallower than the standard mortality curve, more similar to the higher dilutions effect seen in the infectious dose trials. This suggests a lower virus dose effect is present. While it is possible the prawns in the water borne challenge tanks could have been exposed to virus as early as day 0-3 when the oral challenge prawns were fed the infected tissue, it is more likely the major exposure occurred once the prawns in the oral challenge tanks began to die. If that is true it could be speculated that only around 14 days lapsed between challenge and clinical disease. This is a much shorter incubation period than that seen in the oral challenge. WSSV, YHV and Baculoviral Mid-gut Gland Necrosis Virus (BMNV) and Taura Syndrome Virus (TSV) have all been shown to be transmitted via virus contaminated water experimentally (Lotz 1997; Chou *et al* 1995; Maeda *et al* 1998; Flegel *et al* 1997; Momoyama and Sano 1996). It must be acknowledged this trial would not represent the real world situation in an epizootic. It is unlikely prawns would be continuously exposed to a viral challenge (water recirculation) in a small volume of water.

We would still consider the most likely route of horizontal transmission of MCMS in an epizootic to be the oral route. That is to say, sick prawns being eaten by other prawns. This is consistent with what is suggested for YHV (Flegel *et al* 1997). Really the only thing that can be said with assurance is that water borne transmission is possible and that must be considered when developing disease emergency management plans.

7.5 Susceptibility of other crustacean species

The aims of these trails was to determine the susceptibility of important crustacean species to MCMS virus(es).

Methods.

The experimental *Penaeus (Marsupenaeus) japonicus* and *Penaeus esculentus* prawns came from the CSIRO Cleveland Marine Laboratory prawn breeding program. The *P. japonocus* had a mean weight of 6.7 grams and the *P. esculentus* had a mean weight of 7.1 grams. The *Penaeus (Fenneropenaeus) merguiensis*, *Scylla serrata* (mud crabs) and *Portunus pelagicus* (sand crabs) were caught as adults from Cleveland Bay (Townsville) and associated tidal

creeks. The prawns were maintained in 60 litre aquaria with continuous aeration and an internal corner filter containing coral rubble and filter wool. The aquaria containing *P. japonicus* prawns also had a 4 cm deep layer of sand on the bottom. Water temperatures were held at 28 ± 2°C. Water quality was maintained by three 80% water exchanges per week and the filters were cleaned once a week. The crabs were held in 1000 litre flat bottomed, rectangular plastic tanks with continuous aeration and an external biological filter containing coral rubble and bioballs. Water quality was monitored and seawater exchanged as required to maintain total ammonia levels less than 1 mg/l. The prawns were fed an appropriate commercial pellet food *ad lib* but not to excess. The crabs were fed 4 cm pieces of a purchased bait fish *ad lib*.

The ultrafiltrate used in these trials was prepared from second passage infected prawn carcases using the standard method (see previously). After at least one week of acclimatisation in aquaria or tanks, the prawns received 0.05ml of ultrafiltrate or 2PBS injected into abdominal muscle. The crabs were injected into haemolymph via the articulating membrane at the base of the swimming leg.

The experimental design used is outlined in Table 5. Due to the limited numbers in some species and in some trials, the design did not always allow for full replication or controls. The trials were run for 30 days.

Results and Discussion.

Table 5: Susceptibility of marine crustaceans to MCMS virus(es)

Species	Innocula (replicates x number of animals)	Mortalities at Day 30	% Mortality
Trial A			
P. esculentus	MCMS ultrafiltrate (2x10)	15/20	75
	2PBS (1x10)	0/10	0
P. japonicus	MCMS ultrafiltrate (2x10)	19/20	95
	2PBS (1x7)	4/7	57
P. monodon	MCMS ultrafiltrate (1x10)	10/10	100
Trial B			
P. merguiensis	MCMS ultrafiltrate (2x10)	16/20	80
	2PBS (1x10)	1/10	10
P. japonicus	MCMS ultrafiltrate (1x6)	3/6	50
P. monodon	MCMS ultrafiltrate (1x10)	10/10	100
	2PBS (1x10)	2/10	20
Trial C			
Portunus pelagicus	MCMS ultrafiltrate (2x10)	1/20	5
	2PBS (1x10)	0/10	0
Scylla serrata	MCMS ultrafiltrate (1x10)	0/10	0

Table 5 shows the results. There were some problems with *P. japonicus* control survivals, but these survivals were still significantly different compared to challenge survivals to be able to conclude *P. japonicus* are susceptible to MCMS virus(es). Similarly *P. esculentus*

and *P. merguiensis* were also lethally infected by MCMS virus(es). Final mortalities were not as high as *P. monodon*. These results represent the theoretical possibility than other prawn species could be lethally infected by MCMS. The reality is that on the few farms that cultured *P. monodon* at the same time as *P. japonicus* or *P. monodon* and *P. merguiensis*, epizootics affecting the *P. monodon* did not spread to *P. japonocus* not *P. merguiensis*. This probably reflects the mechanism of infection, in that we feel oral transmission is the primary route of transmission on farms once an epizootic has begun. It would have been good to repeat these trials using an oral challenge. Unfortunately due to the difficulty in sourcing healthy *P. monodon* for the other transmission trials, and the need to repeat trails many times to get valid results, time ran out in the project.

The two crab species are not susceptible to MCMS virus(es). This trial indicates experimental MCMS lethal infections are restricted to prawn species similar to most important prawn viruses (see Lightner 1996). It is only WSSV which has a range of hosts across different crustacean families (Lo and Lou 1998).

7.6 The effect of desiccation and chlorine disinfection on MCMS virus(es) viability.

These trials were to evaluate *in vitro* possible control methods for on-farm eradication of MCMS after an epizootic.

Methods.

Two types of successful trials were completed, in the first an ultrafiltrate was used, secondly infected prawn heads were used.

Experimental prawns were obtained from prawn farm ponds with no recent history of ill health. The prawns had a weight range of 7 to 10 grams. The prawns were maintained in 120 litre aquaria with continuous aeration and two internal corner filters containing coral rubble and filter wool. Water temperatures were held at 28 $\pm$ 2ºC. Water quality was maintained by three 80% water exchanges per week and the filters were cleaned once a week. The prawns were fed a commercial pellet food *ad lib* but not to excess. Ten prawns were used in each aquaria.

Trials using ultrafiltrate

The experimental design for the chlorine disinfection was similar to the previous dilution trials. Each treatment was duplicated. There were three control treatments; untreated control, self control (injected with ultrafiltrate prepared from a sub-sample of the group of experimental prawns) and a positive control or the 10^0 dilution (injected with ultrafiltrate prepared from prawn carcases collected from a farm epizootic). Three dilutions (10^{-1}, 10^{-3}, and 10^{-6}) of the positive control ultrafiltrate were used to prepare aliquots for chlorine treatment. These diluted aliquots were treated with chlorine in the form of a 100gCl/L stock solution of sodium hypochlorite. Each of the three aliquots were treated at 200mg/L for 24 hours and 1,600mg/L for 4 hours at room temperature. The chlorine was neutralised at the end of the prescribed time using sodium thiosulfate (0.25 of the treatment Cl_2 concentration). Any precipitant formed by the neutralisation step was removed by centrifugation for 5 minutes at 13,000 rpm. Once prepared, the chlorine treated/neutralised ultrafiltrates were injected into experimental prawns at a dose of 0.05ml per prawn.

In the desiccation trial, ultrafiltrate (prepared from prawn carcases collected from a farm epizootic) aliquots (1 ml) were placed on sterile absorbent filter paper in a 34 °C hot room. All preparations were left for one hour at which time filter paper had become completely dry; this is referred to as Time 0. At this point one filter paper was removed and prepared for viral extraction and labelled Time 0. At 30 minutes post Time 0 another filter paper was removed then labelled Time 30 and again at 240 minutes (4 hours) for Time 240. The dried ultrafiltrate aliquots were then processed by cutting out filter paper around the watermark and cutting this section into smaller 5mm square pieces. These were placed in to 10ml of PBS and held at 4°C overnight. The following day preparations were stirred for 15 minutes and then centrifuged to remove paper leaving a processed ultrafiltrate (at a 10^{-1} dilution). There were three control treatments; untreated control, self control and a positive control (injected with ultrafiltrate prepared as above however filter paper was cut out immediately after the initial ultrafiltrate was placed in the paper and then immediately placed into PBS so no desiccation occurred). Once prepared these processed ultrafiltrates were injected into experimental prawns at a dose of 0.05ml per prawn. Each treatment was duplicated.

Trials using infected prawn heads

The experimental design for the chlorine disinfection trial was similar to the above using ultrafiltrate. Each treatment was duplicated. There were three control treatments; untreated control, self control (injected with ultrafiltrate prepared from a sub-sample of the group of experimental prawns) and a positive control (injected with ultrafiltrate prepared from prawn carcases collected from a farm epizootic). Two prawn heads collected from the farm epizootic were placed in a 1 litre solution of chlorine, either 200mgCl/L for 24 hours or 1,600mgCl/L for 4 hours. Prior to removal of the prawn heads, the chlorine was neutralised using sodium thiosulphate. The heads were then processed in the standard method (see previously) to produce an ultrafiltrate. The experimental prawns were each injected with 0.05ml ultrafiltrate.

The desiccation trial used intact prawn heads from previous trial positive control prawns injected with ultrafiltrate prepared from prawns collected at a farm experiencing MCMS. The heads were weighed and placed in glass aquaria modified to prevent scavenging. The aquaria were placed outside at midday, fully exposed to sunlight and overnight temperatures. The heads were removed at specified times, that is after 24, 72 (3 days) and 168 hours (7 days). Prior to processing to ultrafiltrates, extra PBS was added to account for fluid loss in the prawn heads during desiccation. Three control groups (untreated, self and positive) were run concurrently with treatments using ultrafiltrate prepared from the desiccated prawn heads. All controls and treatments had two replicates. The control and treatment ultrafiltrates were then injected into experimental prawns at a dose of 0.05ml per prawn.

Results and discussion.

As discussed previously, one of the major constraints in the project was the difficulty in obtaining healthy *P. monodon* juveniles from farms for experimental studies. The results for the chlorine disinfection of ultrafiltrate trial presented here are the third attempt, while the other trials all produced valid results on the first attempt.

The results from the four trials are given in figures 7, 8, 9 and 10. The replicates of each treatment were averaged and plotted.

In the chlorine disinfection of ultrafiltrate trial there were some water quality problems in one of the replicates receiving the 10^{-6} at 1,600mg/L chlorine treated/neutralised ultrafiltrate causing a slight reduction in survival at 30 days (Figure 7). With that exception, the results clearly indicate that even at the high dose (10^{-1}) the two chlorine disinfection protocols used were completely effective in inactivating the MCMS virus(es).

Surprisingly the desiccation of ultrafiltrate upto 240 hours did not appear to have any effect on MCMS virus(es) viability at all. While the untreated and self control survivals were 80 and 65% at 30 days, there was a significant difference from positive control and treatment survivals indicating the results were valid. All the mortality curves were not as steep as the standard mortality curve. This possibly indicates some virus(es) is removed when ultrafiltrate is placed on filter paper, no matter whether there is subsequent desiccation or not. The curves seen in Figure 8 may represent a lower initial virus dose on injection challenge.

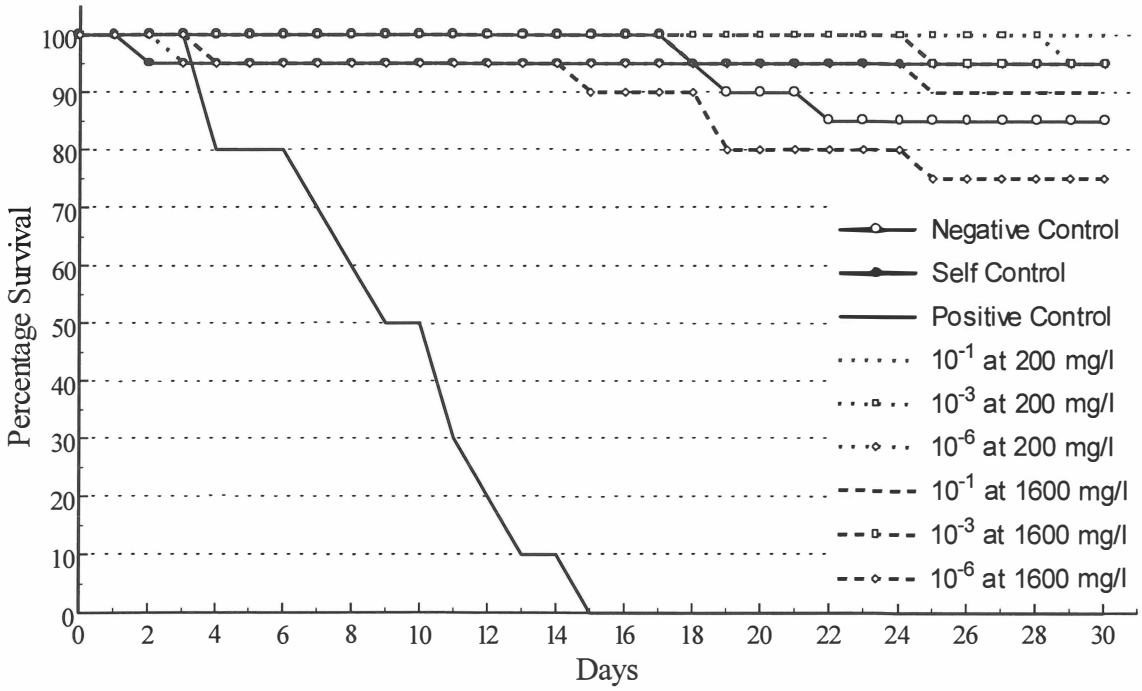

Figure 7: Mortality of experimental prawns following chlorine (as a solution of sodium hypochlorite) disinfection of MCMS ultrafiltrate (mean data from replicated tanks).

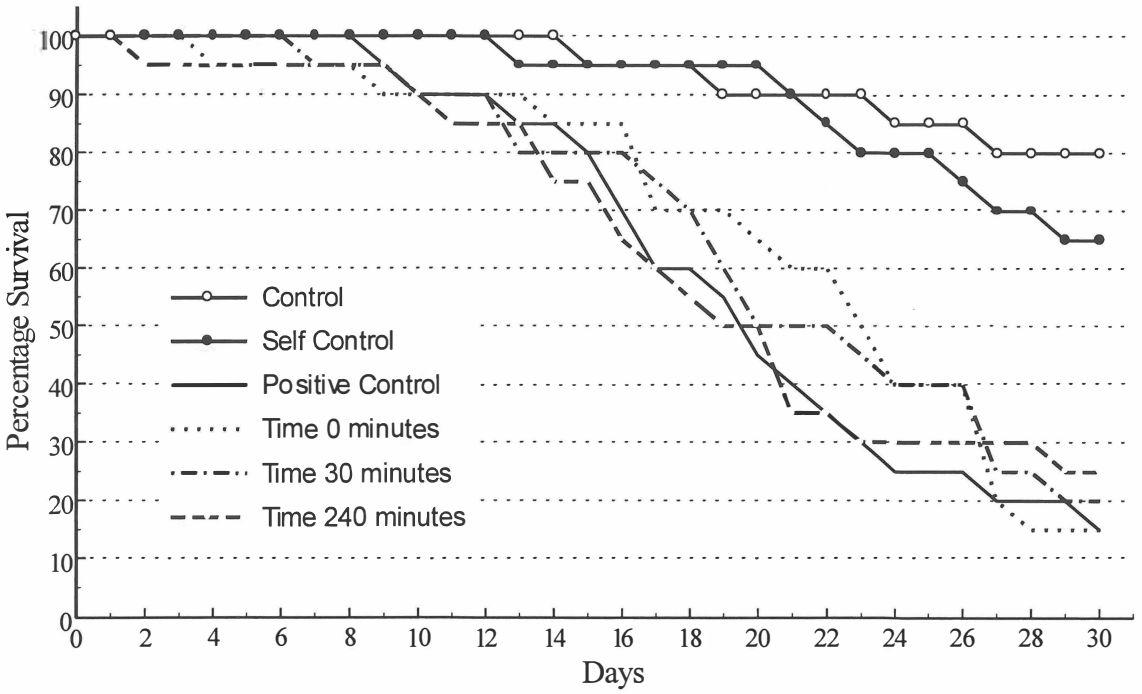

Figure 8: Mortality of experimental prawns following desiccation of MCMS ultrafiltrate (mean data from replicated tanks).

The trials using infected prawn heads were meant to represent a more realistic situation. The tissue would provide some protection against desiccation, while the chlorine would be deactivated when exposed to organic materials in the prawn heads, leaving less free chlorine to destroy the virus(es). Figure 9 shows the results of chlorine disinfection. Again, even though untreated and self control survivals were on the low end of what we would prefer,

there was a significant difference between the positive control mortality curve and the other treatment mortality curves. The results show that either chlorine disinfection regime, and probably lower chlorine concentrations, would effectively inactivate MCMS virus(es).

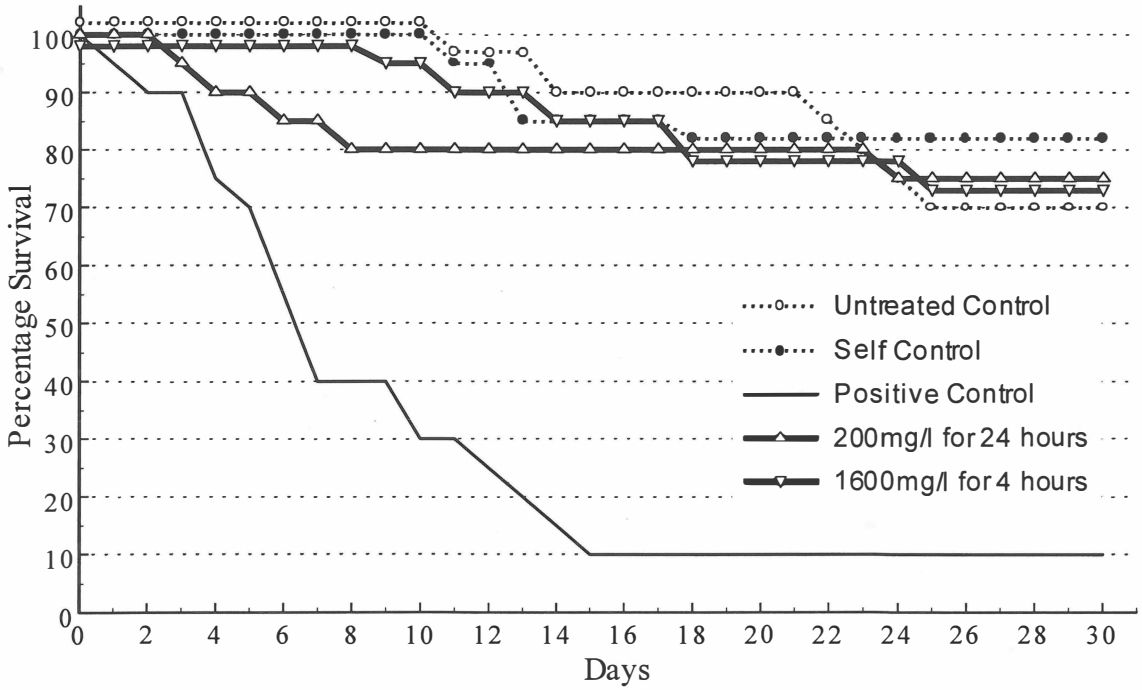

Figure 9: Mortality of experimental prawns following chlorine (as a solution of sodium hypochlorite) disinfection of infected prawn heads subsequently prepared as ultrafiltrate (mean data from replicated tanks).

The results from the desiccation of prawn head trials was again surprising. The period of exposure to sunlight and desiccation was extended as compared to the trial with ultrafiltrate. Figure 10 shows the results after ultrafiltrate was prepared from desiccated prawn heads. Untreated and self control survivals were above 80% at 30 days. The treatment survival curves were significantly different from the positive control mortality curve. But the survivals of 60 to 70% suggest that, even after one week (168 hours) of environmental exposure there appeared to be some viable virus(es) remaining in the prawn head. Clearly the shallow mortality curves observed indicate a much reduce viable virus dose, but more than one week would be required to completely inactivate the MCMS virus(es).

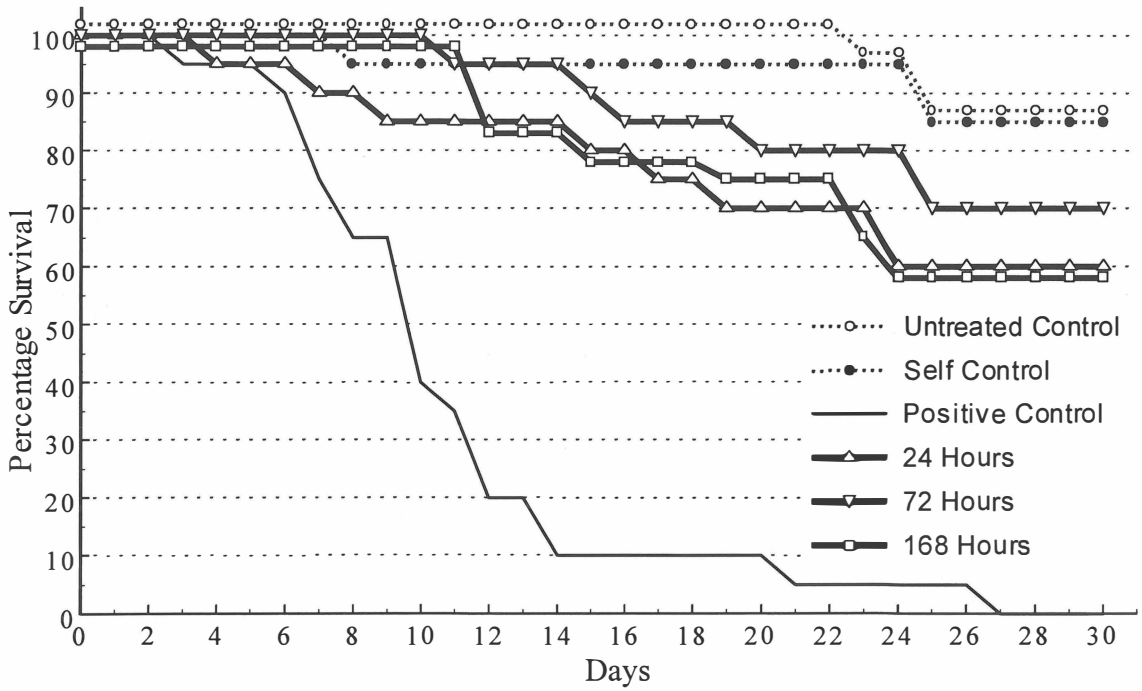

Figure 10: Mortality of experimental prawns following desiccation of MCMS ultrafiltrate (mean data from replicated tanks).

Few comprehensive research reports are available to describe prawn virus deactivation by chemicals or physical conditions. The most comprehensive information has been published by Momoyama (1998a and b) using purified suspensions of BMNV. This research found 1.5 hours of drying at 30°C or 5mg chlorine/l, 25mg iodine/l, 0.5% formalin and 100mg benzalkonium chloride/l for 10 minutes were sufficient to destroy BMNV. Again using an ultrafiltrate (or purified suspension of virus), Maeda *et al* (1989) reported inactivation of WSSV by 10mg chlorine or iodine/l for 30 minutes, heating to 50°C for 20 minutes or drying at 30°C for one hour. Less specific information has been given by Flegel *et al* (1997), that is '30mg chlorine/l is an effective disinfectant for YHV'. 70ppm formalin is said to destroy WSSV in water (Flegel *et al* 1997). Dixon and Dorado (1997) indicate Bell and Lightner's (1992) clean-up recommendations will destroy TSV eg., chlorination ensuring a minimum residual free chlorine at 10mg/l after 24-48 hours.

The projects use of entire, infected prawn heads more closely represents the situation on prawn farms after a MCMS epizootic. From our trials, we would not recommend short (1-2 week) pond dry-outs as an effective method to destroy MCMS virus(es) as could be implied from reference to overseas research reports. To the contrary, chlorination appears highly effective. It is possible even lower concentrations than those used in these trials would be effective. The technique of flooding ponds with seawater then adding liquid chlorine has been used by Queensland farmers to disinfect ponds after MCMS epizootics. This trial supports the use of chlorine (at 100-200mg/l for 24 hours) in this manner as a highly effective approach to elimination of MCMS virus(es).

8. Farm monitoring and analysis

8.1 Farm diagnostic records

QDPI veterinary laboratories use a relational data base (LOIS: Laboratory On-line Information System) to record all laboratory analyses created from diagnostic case submissions. On submission, basic clinical information is recorded eg., age of prawns, species of prawn, local weather conditions, status of pond water quality and appearance of diseased prawns etc. LOIS provides valuable historical information on animal diseases in Queensland and is used by veterinary managers to develop and monitor state animal health programs.

Methods.

The data base was interrogated on a case by case basis. Key information was recorded in tables which included relevant histopathological lesions. The juvenile prawn submissions were then summarised to determine qualitative trends over time. For the purposes of this analysis, MCMS was defined as those cases/ponds which had a clinical history of significant mortalities preceded by a marked reduction in feeding but had no obvious aetiology eg., bacterial septicaemia or haemocytic enteritis. Suspect MCMS were those cases/ponds which did not necessarily have significant mortalities but did have un-attributable histopathological lesions which included; intra-nuclear inclusions in anterior midgut caeca, hypertrophied nuclei in gill epithelium or heart cells with no nucleoplasm, and/or focal necrosis of the ventral nerve cord or thoracic peripheral nerves. The quality of this information is not as great as properly designed epidemiological surveys, as not all prawn farms with disease problems will submit prawns from affected ponds. Despite this limitation some useful observations can be made.

Results and Discussion.

Table 6 presents a summary of some of the LOIS information. There clearly was a peak in MCMS problems through the 1995/96 and 1996/97 seasons. By 1997/98 most affected farms had undertaken a total farm destocking and, in most instances, a chlorine disinfection of ponds and canals. The diagnosis of MCMS or suspect MCMS in 1997/98 comprised submission of prawns from just two farms. In fact the majority came from only one farm. This was the only farm which never did a complete destocking of their entire farm complex. By the 1998/99 season MCMS ceased to be a problem, although a suspicion remains that some farms may have problems and do not report nor submit prawns for diagnostic investigation. Another interesting feature of the prawn farm disease problems, was that by the end of the 1996/97 season, MCMS outbreaks typically occurred close to pond harvest. That is to say, it stopped being a mid-crop problem. There was a close association between outbreaks and maximum pond biomass and, usually, poor intake/pond water quality due to climatic events.

Other than this latter manifestation of MCMS, we were never able to consistently identify any specific pond stressor that would precede an outbreak. Even detailed analysis of pond cyanobacterial (blue-green algae) blooms failed to directly connect cyanobacteria with the onset of MCMS (C. Robertson, pers. comm.). Quite successful crops, with no MCMS, can be produced in ponds which had dominant cyanobacterial blooms during the production

cycle. Although persistent cyanobacterial blooms in a pond do appear to affect the 'health' of prawns. We proposed a hypothesis, for MCMS to occur one or more viruses must have a critical level of prevalence in a pond population (and probably a high intensity of infection in individual prawns) and that two or more different stressors need to act at the same time on that population of prawns. The stressors could be any one of a range of factors, for example, dissolved oxygen levels, pH variation, cyanobacterial blooms, phytoplankton bloom collapse or poor pond bottom conditions. Any one occurring alone would be insufficient to create the stress required to 'trigger' a MCMS outbreak.

Table 6: A summary of MCMS incidence in submitted prawns to QDPI and the presence of LOV/GAV and SMV in clinically diseased prawns.

Season	Number of Ponds (Cases[1])	MCMS or suspect MCMS[2]	Covert GAV (LOV)[3]	Overt GAV[3]	SMV[3]
	From which prawns were submitted to QDPI for diagnosis	Number of ponds	Positive ISH reaction to lymphoid organ spheroids	Positive ISH reaction to tissues (not lymphoid organ)	Positive ISH reaction (all reactions were weak)
1994/95	32 (16)	22	4/10	1/15	1/1
1995/96	83 (51)	33	3/16	1/19	4/4
1996/97	82 (33)	22	10/14	4/19	8/12
1997/98	84 (46)	29	9/16	1/21	2/3
1998/99	31 (29)	1	ND[4]	ND	ND

[1]Cases = a single submission which is from one farm and may include prawns from one or more ponds.
[2]suspect MCMS = those ponds where diseased prawns have unexplained tissue lesions which could be caused by viral infections.
[3]*in situ* hybridisation detection tests were done on selected archived prawn tissues (stored as histological blocks) from ponds with MCMS or suspect MCMS.
[4]ND = not done.

8.2 SMV and LOV/GAV in archival prawn tissue from diagnostic cases.

Towards the end of the project *in situ* hybridisation (ISH) tests became available for Oonoonba Veterinary Laboratory (OVL) use. It was planned to determine the relative significance of the two principle viruses, thought to be involved in MCMS, in prawn tissues stored at OVL from MCMS outbreaks. Testing of archival prawn tissues at JCU with the SMV ISH (section 6.2 of this report) found that SMV was not always present in prawns from MCMS outbreaks. This suggested a more significant role for LOV/GAV. K. Spann (CSIRO Indooroopilly, CRC Aquaculture project A.1.4) was able to provide the probe and methodology of the ISH she developed for LOV/GAV. This provided the opportunity to test archival prawn tissues held at OVL from MCMS outbreaks for LOV/GAV and SMV.

Methods.

Prawn tissue selection: All prawn cases were reviewed. Tissue blocks from 1991 onwards were retrieved from archival storage. Tissue blocks which contained longitudinal, midline sections of a prawn thorax were used. A representative prawn from a pond with MCMS or suspect MCMS was selected from the 1994/95, 1995/96, 1996/97 and 1997/98 seasons. Some prawns from 1991 and 1992 were also ISH tested to assess preservation of intact viral nucleic acid, particularly RNA. Not all thoracic sections selected contained lymphoid organ hence the total number of sections examined for overt GAV is greater than for covert GAV (LOV).

LOV/GAV ISH: The methodology provided by K Spann (pers. comm.) was followed. Interpretation of probe positive reactions also followed that recommended by K Spann. This is summarised by; with *in situ* hybridisation, covert GAV (LOV) infection is restricted to spheroids within the lymphoid organ; while covert GAV occurs throughout the lymphoid organ, including apparently normal tubules, and in various connective tissues and gills.

SMV ISH: The methodology, using the labelled 400bp probe, developed in this project at James Cook University was followed. Any probe reaction with the prawn tissues in the selected sections was considered a positive reaction.

Results and Discussion.

Positive GAV ISH reactions were seen in lymphoid organ spheroids in prawns from 1991 and 1992. Positive SMV ISH reactions were also seen in prawns from 1991 and 1992. This indicates that covert GAV and SMV infections have been around for some time in cultured *P. monodon* in Queensland. It also suggests that the routine procedure used for preserving diagnostic prawn specimens at OVL does not cause complete GAV RNA degradation.

As a general comment, not all lymphoid organ spheroids would give a GAV ISH positive reaction. Even in the same prawn, some spheroids would be positive while others remained unstained. This can either be interpreted as different viruses in the same prawn cause spheroid formation or that low levels of GAV in some spheroids are not sufficient to cause a detectable probe reaction. Even in prawn tissue from 1998 some spheroids did not give a positive GAV ISH reaction. Complete acceptance of the results from using the SMV ISH needs some care. There was a limited amount of labelled probe available for the SMV testing of the selected, archived prawn tissue sections. The SMV ISH reactions seen were weak and involved a variety of tissues eg., sub-cuticular connective tissue, muscle, lymphoid organ spheroids and hepatopancreas.

A summary of the LOV/GAV and SMV ISH results for the 1994/95 to 1997/98 seasons are given in Table 6. There appears to be no consistent GAV ISH positive reaction to lymphoid organ spheroids. In none of the prawn tissues did a generalised positive reaction to the GAV ISH occur in the lymphoid organ. Similarly, very few systemic prawn tissues gave a positive reaction as expected if they had an systemic, covert GAV infection. In fact none of the prawn tissues tested from MCMS or suspect MCMS outbreaks fulfil the criterion proposed by Spann (pers. comm.).

There may be storage effects on the GAV RNA and SMV DNA. The consistent positive reactions in tissues as far back as 1991 tends to indicate that, while RNA/DNA degradation on storage may reduce the sensitivity of the ISH detection tests, there remains sufficient intact viral nucleic acid for the probes to hybridize with to produce a detectable reaction. The GAV ISH results could well indicate, that like SMV, GAV is not always present in cases of MCMS. The result is that we still need to be careful how we interpret research results. The actual situation in clinical disease on farms appears much more complex.

8.3 Cyanobacterial toxicity

Cyanobacteria have been associated with prawn disease and many farmers and researchers suggested that they might be playing a role in MCMS. Cyanobacteria are reported to cause haemocytic enteritis (HE) (Lightner 1985). The associated endotoxin has to be released by rupturing the cyanobacterial cell. Therefore, a histopathological survey of 461 prawns from three ponds was undertaken for HE in conjunction with cyanobacteria in the gut or as a fouling organism on the gills (Young 1997). The relative risk of HE with cyanobacteria in the gut was 1.16 ($P>0.05$) and in the gills it was 2.55 ($P>0.05$), suggesting no statistical link.

To further examine the role of cyanobacteria in MCMS, the prawns ability to survive toxicity was examined (Snape 1998). Smith (1997) identified *Nodularia* as a hepatotoxin-producing cyanobacteria found in prawn ponds. Therefore, an experiment to measure the lethal dose 50% (LD_{50}) of nodularin for *Penaeus monodon* was conducted. In two experiments, no prawns died at the highest dose, 300mg/kg. This dose is five to six times the LD_{50} for mice. Histological examination of the prawns showed no changes consistent with nodularin toxicity in the hepatopancreas or heart and no HE. This suggests *Penaeus monodon* have a high resistance to the cyanobacterial toxin, nodularin.

9. Development of routine screening procedures

SMV has been associated with mortalities in broodstock of *Penaeus monodon* and with MCMS on growout farms. Epidemiological evidence suggested a major link between the broodstock and progeny postlarvae, which led to on-farm mortalities. Therefore, a routine screening procedure was devised and ground-truthed as far as possible. The faeces of 909 broodstock were tested by a PCR for SMV and confirmed by an internal dot blot. Seventy-seven spawners (8.5%) across two species were positive for SMV but prevalence ranged from 0 to 24% (Table 7). Spawners of *P. monodon* were more highly positive (24%) than were spawners of *Penaeus merguiensis* (4%). Due to hatchery management considerations, progeny of infected *Penaeus monodon* spawners were mixed with progeny of non-infected spawners at various concentrations and their survival monitored in both the hatchery and the growout ponds. Statistical analysis of regression lines showed survival was negatively correlated to contamination rate and that 23% less progeny survived in the hatchery from infected females (51% survival) than from uninfected spawners (74% survival) (adjusted R^2=0.27, P<0.01). Analysis of two other independent data sets of 189 batches of penaeid larvae demonstrated statistical significant (P<0.01) losses of 15% and 24% respectively. Data from 38 ponds suggested that 15% of losses were due to SMV. Two ponds of newly stocked postlarvae were followed for six weeks in growout ponds using a SMV gene probe. Mortalities to SMV were estimated to be 20% and 35% respectively. The loss to the industry due to SMV is estimated to be at least $4.6million per year. This information has been submitted for publication (Owens et al submitted).

Table 7: The prevalence of SMV-infected spawners at hatcheries detected by PCR/dot blot on faecal samples.

Date	No of Positives/No of Broodstock	Prevalence (%)
12/97	7/30	23
1/98	1/102	1
2/98	7/70	10
3/98	10/126	8
8/98	6/138	4
9/98	8/108	7
12/98	7/176	4
9/99	31/131	24
9/99	0/28	0
Totals	**77/909**	**8.47**
P. monodon	65/266	24.4
P. merguiensis	12/277	4.33

Based on the rigorous data summarised above, a screening program targeting the faeces of spawners appears possible and is as follows.

Faecal Collection. Faecal samples were boiled immediately after collection in TE buffer (20 mM Tris-HCL, 10 mM EDTA at pH 8.0) as laboratory studies had shown that nucleases

present in the faeces quickly destroyed any viral DNA present. The samples were then stored at –20°C until further processing.

DNA Extraction from Faeces. The method used to extract DNA required the exclusion of melanin from the extract which inhibits PCR. Samples were vortexed to disrupt the faecal strings and digested with 200ug/ml proteinase K (PK) and triton X-100 for 2hrs at 56°C. The DNA was then selectively attached to silica and proteins denatured with a chaotropic agent (NaI) (Progen Industries, Bandpure™). The nucleic acid extract was washed and eluted as per manufacturers instructions. The DNA was resuspended in a final volume of 30ul of 10mM Tris-HCl pH 8.0. Five microlitres of the extract was used in a 25:1 PCR reaction.

Testing of Spawners. A set of PCR primers (SMV200) and a DIG-labelled dot blot confirmation test (SMV150) for one of the amplicons were designed from a cloned piece of SMV DNA. Experience showed that SMV200 with the internal dot blot (SMV150) was the most robust. This test would detect about 100 copies of the genome of SMV. Each faecal sample was run in duplicate to make sure the number of false negatives was limited.

One person can extract the DNA, run the duplicate PCRs and do the confirmation tests for 100 spawners in about 4 days, using the currently available technology. It takes another day to prepare reagents and sterilise the equipment for the next batch. The time-consuming procedure is the critical DNA extraction and it is anticipated improvements in extraction methodologies associated with commercial kits will give considerable savings in time.

The testing of postlarvae for SMV by PCR has not been considered a success. Melanisation has consistently interfered with the PCR by either blocking it completely, or by producing multiple spurious bands as well as the SMV diagnostic band. With further development, it is felt that a methodology could be devised. Critical points to consider are: the postlarvae samples should be stored in 70-90% alcohol; the eyestalks of each postlarvae needs to be cut off before the DNA extraction is undertaken. The second point suggested by Prof. TW Flegel has not been tested by us.

10. Proposed screening program

10.1 Is it epidemiologically sound to test for SMV?

Since only 24% of female spawners are infected with SMV, it is practically possible to get enough SMV-free animals to stock all growout ponds. This view is enhanced by some batches of broodstock being free of SMV. With time, the effect of geographical areas and season on SMV will become clear and appropriate targeting fishing of SMV-free broodstock may be possible.

10.2 Is it economical to test for SMV or not?

The market value of the harvest of Queensland *P. monodon* was worth around $24.8 million in 1998/99 (this figure includes *P. esculentus* and *P. merguiensis* production; Lobegeiger 2000). Simplistically, if a minimum of 15% of production was lost to SMV, then approximately $4.7 million was lost in growout and a further $0.5 million was lost in hatcheries (based on the figures in Lobegeiger 2000). This gives an annual loss of $5.2 million to the *P. monodon* industry, which does not include wage costs nor lost opportunity costs in hatcheries. The laboratory costs for PCR testing of all the spawners for the entire industry plus replacement costs of SMV-PCR-positive spawners are estimated at $220,000-$250,000. This does not include wage costs of collecting spawner faeces and processing it in the hatchery. These figures give a minimum multiplier to production of approximately 16 times for every dollar spent in testing. So in terms of potential economical benefit, testing seems justified. However, due to the period of intense demand for testing services during the major stocking periods, further development of the SMV PCR into a kit form is necessary to cut down turn around time.

However, the extra costs in time and labour; the housing of individual spawners whilst waiting on test results plus the opportunity costs due to discarding SMV-positive spawners are all met by the hatchery operators. But the big monetary benefit is gained by the growout portion of the industry. Unless some method of payment makes it worthwhile for the hatchery operators, a testing program will not proceed or it will be so poorly executed as to be useless. One method of recompense is for growout farmers to pay a premium price (3^{c} ?) for postlarvae tested and shown to be free from SMV.

10.3 Parallel testing for other viruses?

It is theoretically possible to multiplex the SMV PCR to test for other viruses, particularly those that are shed in the faeces ie (HPV and monodon baculovirus (MBV)). This would give the industry a higher level of biosecurity. It would need some modification of PCR primers and protocols to ensure compatibility. However, current data on the biology of these viruses suggests that this is unjustified. HPV has not been a problem in *Penaeus monodon* in northern Queensland (Kahn *et al* submitted). Similarly, there is no reliable data that suggests MBV has been a problem in *P. monodon*. Furthermore, the testing of faeces for whitespot syndrome bacilliform virus (WSSV) as a preventative measure is not appropriate, as this systemic virus has not been demonstrated in the faeces. Similarly, the validity of testing for the systemic gill-associated virus (GAV) in the faeces has not been ascertained. Moreover, given that the GAV infection rates are reported to be 98%, a practical strategy for stocking the entire industry with GAV-free prawns is yet to be devised. In summary, with the current level of knowledge, the faecal screening for multiple viruses in addition to SMV does not appear to

be worthwhile. It is anticipated that changes in knowledge and advances in the sampling of tissues for other viruses may lead to this concept being re-addressed in the future.

10.4 Practicalities of faecal testing for the industry

Evidence from one hatchery suggests that eyestalk tagging of spawners as their faeces were collected led to stress and regression of their ovaries. Therefore, as trialed by a different operator, it seemed better to individually house the spawners in 20L, flow-through containers within the larger maturation tanks whilst awaiting the results of the SMV PCR. Spontaneous spawnings are more problematical as there may not be enough nauplii-raising tanks to keep them all separate. Obviously in this case, the less mixing of broods the better, as the amount discarded needs to be limited.

With further development, the possibility that a single laboratory could undertake the SMV PCR screening for the entire industry seems almost feasable but not desirable. In 1998, 3368 spawners were used (Lobegeiger 1999) and anticipating a rejection rate of 24%, 4176 spawners would be needed. Assuming the spawners are used approximately equally for the spring and the post-Christmas stocking, 2,088 would need to be processed over a 6-8 week period; a maximum of 348 spawners/ week would have to be processed. By having two people undertaking the DNA extraction, the time-expensive step can be curtailed. However, due to the period of intense demand for testing services during the major stocking periods, further development of a ELOSA kit would allow the PCR and confirmation test to be a single step process, thus saving an additional day. Furthermore, development of a SMV PCR kit would allow farmers to use independent testing laboratories with which farmers can build up their rapport and ensure confidentiality.

11. Farm and hatchery clean up procedures.

The evidence from the live virus infection trials indicate MCMS could be transmitted via feeding on infected tissue and via the water. These are two important factors to consider in control and prevention strategies. Importantly, the likely source of SMV and GAV (P. Walker, pers. comm.) are broodstock. This information implies hatchery broodstock facilities require particular attention in clean-ups. From information produced in this project, and from overseas data on WSSV and YHV, it is clear disinfection should involve chlorine solutions (or equivalent halide ion solutions).

Farm Clean-up.

This procedure particularly applies following an MCMS outbreak, but is recommended as a standard annual practice for all prawn farms. Elements of Bell and Lightner's (1992) clean-up recommendations are included in the following protocol. Also refer to Lightner and Redman (1998). It is important farmers understand it can only take a few viable viruses to re-establish a disease. From the trials done in this project, an infectious dose can be found in as little as 0.125µg of prawn tissue (a minute amount). If the clean-up procedure is not done completely, any partial effort becomes a waste of time and money. Effective farm clean-up requires careful planning and organisation.

1. Remove all prawn carcases, prawn wastes and organic material from ponds, access areas and canals.
2. Completely destock entire farm complex.
3. Remove all equipment, furniture and materials from ponds (including pickets and removable posts).
4. Clean equipment and permanent pond structures with high pressure water washing.
5. Thoroughly dry ponds, canals and equipment to kill fouling organisms. Clean the equipment again with high pressure water.
6. Disinfect the ponds and canals by flooding the floor with at least 200mm of clean seawater, add sodium hypochlorite to get a 100mg/l free chlorine concentration and leave for 24 hours. The aerate for a further 24 hours to dechlorinate. Test for Total Chlorine in the seawater, discharge only if no residual chlorine can be detected.
7. Spray pond/canal walls, permanent pond structures, access areas and equipment with a concentrated (1,600mg free chlorine/l) sodium hypochlorite solution. Allow to dry for at least 24 hours. Equipment or materials sensitive to chlorine may be disinfected with phosphonic acid-free PVP-iodine solutions (eg., Betadine®) using a 1,600 free iodine/l concentration.
8. The entire farm complex should remain dry and destocked for at least 4 weeks.

Alternative pond disinfectants are recommended by Bell and Lightner (1992), but we found that the MCMS virus(es) are highly susceptible to chlorine disinfection and recommend that as the disinfectant of first choice.

Hatchery Clean-up.

Standard hatchery clean-up procedures to maintain microbial hygiene are critical to ensure sustained hatchery performance and avoidance of luminous vibriosis (see Harris 1997). From the research done in this project, it is clear SMV is present in the faeces of spawners and

particular attention, over and above routine hygiene practices in live food and larval rearing sections, needs to be put on maturation and spawning facilities in the hatchery.

Egg collection devices which have continuous flowing clean seawater are the preferred egg collection technique in hatcheries. This will help limit any accumulation of broodstock faecal material in the spawning section.

Specific clean-up procedures should apply to the broodstock and spawning sections after each batch of broodstock have been used. If the hatchery does not use a batch production system, the clean-up should be applied at least twice a season.

1. Completely destock maturation unit.
2. Dismantle tanks, pipes, recirculation components and equipment to allow effective cleaning. Remove sand and biofiltration materials if required. Dispose of small airlines and air diffusers.
3. Detergent wash tanks (scrub to remove biofilms), equipment, floors (particularly drainage areas and corners where prawn-derived organic material may accumulate) and walls affected by water splash.
4. Thoroughly rinse above in freshwater and dry (moving tanks and equipment outside to sunlight if possible).
5. Fill all pipes and tanks with water with sodium hypochlorite added to 60-100mg free chlorine/l for 60 minutes or 20mg chlorine/l for 24 hours.
6. Place all equipment in 60-100mg chlorine baths for 60 minutes.
7. Spray tank exteriors, walls and floors with 1,600mg chlorine/l and leave for at least 4 hours.
8. Any materials sensitive to chlorine may be disinfected with phosphonic acid-free PVP-iodine solutions (eg., Betadine®) using a 100mg or 1,600mg free iodine/l concentration as appropriate.
9. Rinse all above with or in freshwater after disinfection process and hard dry.

Similar procedures can be used for all parts of the hatchery as part of standard hygiene practice.

12. Benefits

The project had a clear focus, Mid-Crop Mortality Syndrome (MCMS) and its effects on prawn aquaculture in Queensland. Two specific and sensitive molecular detection tests for one of the viruses associated with MCMS have been developed. The tests have been used to demonstrate their applicability to screen broodstock and in diagnostic investigations. While broodstock testing to identify SMV carriers has been trialed in a commercial hatchery, the technique is not yet widely available to industry. The foundation, and evidence of benefit for its broader application, is established. As stated in the project application this will directly benefit the prawn aquaculture industry.

The decline in MCMS incidence as a significant, widespread problem is clearly beneficial to prawn farm profitability. While important questions remain before a scientific explanation can be given for the appearance of MCMS then its decline, information on the source of SMV and how MCMS viruses behave can be used by industry. We now know SMV and GAV have been infecting cultured prawns for sometime (at least since 1992). The absence of major exotic prawn viruses has been demonstrated. This may assist industry access to export markets. The total value of marine prawn production in Queensland has increased from $15.8m (1097.9 tonnes) in 1996/97 to $24.8m (1701.8 tonnes) in 1998/99. This is partly due to the on-farm prevention strategies of total destocking and pond disinfection.

Scientific evidence produced in this project will make control and prevention advise more specific. Current prawn health extension advise by the principal diagnostic service providers to prawn farmers includes information generated by this, and other projects, on prawn viral diseases. Already two farm health management workshops have been run by QDPI in north Queensland. It is hard to quantify all these effects, but a heightened awareness on best practice health management will continue to influence the probable course and industry-effects of MCMS-like epizootics in the future.

13. Further development

Further funding (possibly from the AFFA program – Building a national approach to animal, aquatic animal and plant health) will be sort to develop an ELOSA test and to provide a stronger base for commercialisation of the PCR test for SMV. This is required to reduce laboratory testing times and to make the technology widely available for laboratory use. Refinement of the PCR procedure so it can be used on post-larvae would be desirable.

Farm workshops, extension materials and disease emergency protocols will continue under QDPI prawn health extension activities. These will be used to disseminate the prevention and control information on MCMS.

Further research is required to understand the aetiology and pathogenesis of MCMS. From the results of this project, it appears the viruses SMV and GAV do not always have a clear role in all MCMS outbreaks. More specific knowledge is required on individual-virus and mixed-virus pathogenesis. *In vitro* production of single viruses on cell lines would be of great help in this regard. Epidemiological surveys on wild prawn resources for the known endemic viruses, using the molecular detection technology now available, could identify virus-free stocks for closed-cycle breeding programs. Research on the prawn immune system, particularly on how the pond environment influences its function and how this affects host-virus interactions, is needed before we can understand how MCMS outbreaks start in the first place. It may be that industry will have to continue to operate in the presence of SMV and GAV infections. In this context, the ability to prevent, or at least predict, the situations or circumstances when infections become pathogenic may be critical to ensure the sustainability of prawn aquaculture in Australia.

14. Conclusion

The first Mid-Crop Mortality Syndrome (MCMS) epizootics were seen on some northern prawn farms in 1994. MCMS became more widespread, affecting many farms over subsequent years to peak in the 1995/96 and 1996/97 production seasons. The diagnosis of MCMS or suspect MCMS in 1997/98 comprised submission of prawns from just two farms. In fact the majority came from only one farm. By the 1998/99 season MCMS ceased to be a common problem, although suspicion remains that some farms have disease problems and do not report nor submit prawns for diagnostic examination. Initial investigations on MCMS found at least four different viruses (identified by electron microscopy) were present in diseased prawns. Initial purification to prepare refined suspensions of viruses at JCU identified a small parvo-like virus (now thought to be related to parvo and denso viruses) naming it Spawner-isolated Mortality Virus (SMV). Another virus, also considered to be involved in MCMS, was identified by Dr K. Spann (CSIRO Livestock Industries) and was named Gill-associated Virus (GAV). This project focused on developing and refining the molecular detection tests for SMV.

The first objective of the project was to refine the molecular detection tests for SMV. This was achieved. Initially the PCR test used a primer set (SMV260) which was quite rich in AT and made it prone to produce spurious bands following PCR. Following sequencing, another primer was designed (SMV200). A DIG labelled dot blot for the SMV200 amplicon was also developed as further confirmatory step. The SMV200 primer with the internal dot blot (SMV 150) was found to the most robust method. This combination of tests would detect about 100 copies of the SMV genome. Early pathological analysis determined SMV is primarily an enteric infections in natural outbreaks. Thus the PCR test has been refined to specifically detect SMV in spawner species. Targeting faeces as the sample was desirable as it allows repeated sampling of broodstock, without damage, and can be used in a hatchery screening program. The PCR test cannot yet be used to detect SMV in post-larvae because of interference with tissue components. Further refinement of the PCR test is required if post-larvae are to be sampled.

The *in situ* hybridisation (ISH) test has also been refined. The first probe used was an entire 2000bp DNA insert. A 400bp fragment from the larger insert was found to have better tissue penetration and had less chance of nonspecific binding. The DIG-labelled 400bp probe has been used successfully on a range of archival prawn tissues and on freshwater crayfish tissues.

The second objective was to clearly define the risk factors associated with clinical expression of MCMS. Analysis of archival prawn tissues from disease outbreaks failed to find SMV consistently present in MCMS outbreaks. Use of the GAV ISH (provided by K. Spann, CSIRO Livestock Industries) also found that GAV was not always present in MCMS outbreaks. Both viruses have been present in farmed *P. monodon* since, at least, 1992. The DiagXotics ISH detection test kits for exotic prawn viruses were imported and used on archived diseased prawn tissues (including prawns from MCMS epizootics). The kits recognised MBV and HPV viral infections in Australian prawn tissues. The results were negative for WSSV, IHHNV, TSV, YHV, and BP. The analysis of clinical records failed to identify any single, specific stressor that would initiate a MCMS outbreak. Interestingly, by the end of the 1996/97 season, MCMS outbreaks typically occurred close to pond harvest, not just in the middle of the crop. At these times the outbreak was associated with maximum pond biomass and poor intake water quality and/or an adverse climatic event. Specific

research on cyanobacterial (blue-green algae) toxicity failed to find the cyanobacteria toxins had any effect on juvenile prawns. As a consequence it is still not clear why lethal infections (MCMS epizootics) occurred in 1994 then stopped causing significant problems by the 1998/99 season. As a consequence it has not been possible to accurately define the risk factors for MCMS. We proposed a hypothesis; for MCMS to occur one or more viruses must have a critical level of prevalence in a pond population (and probably a critical intensity of infection in individual prawns) and that two or more different stressors need to act at the same time on that population of prawns. Further research is required to understand the role of different viruses and environmental factors that trigger MCMS-like epizootics.

As it was found SMV originates in broodstock, the establishment of a screening program objective was met with application of a PCR testing of faecal samples in a commercial hatchery. 77 of 909 spawner faecal samples tested positive for SMV by the PCR test. The prevalence of SMV in batches of broodstock varied from 0 to 24%. Regression analysis showed survival of progeny was negatively correlated to the 'contamination rate' in broodstock pools. Data from 38 ponds indicated 15% of prawn production loss could be attributed to SMV. This research indicates it is feasible to screen for SMV by sampling the faeces of broodstock. While further tracing of infections in individual broodstock and their progeny would be desirable, the basis of a screening program to eliminate SMV from prawn production systems by testing for broodstock infected by SMV has been established.

While considerable efforts to purify viruses from MCMS diseased prawns were made we could not achieve this and use single viral suspensions for infection studies. As a result, experimental infections used viral preparations made from carcases of diseased prawns and probably contained a mixture of viruses. Research using live virus(es) addressed a number of aspects of MCMS viral biology required to meet the final objective of determining a program to eradicate MCMS virus(es) from farms and hatcheries. MCMS virus(es) are highly infectious. Very low doses (0.05ml of a 1:1,000,000 dilution of tissue) would cause lethal disease when injected. Experimetal infections were achieved by feeding infected prawn tissue and by sharing water between diseased and healthy prawns. This indicates farms must control ditributuion of sick prawns, or their tissues (eg., control scavenging birds), and water transfer (eg., aerosol drift from paddle wheel aerators) when trying to control a MCMS outbreak starting in one or two ponds. Clean-up procedures need to remove any prawn carcases and all live prawns that may be infected by the MCMS virus(es).

Experimental infections demonstrated that other prawn species were susceptible to MCMS by injection challenge, but that crabs were not. In practice, in on-farm outbreaks, this experimental susceptibility has not been seen. When more than one species of prawn is cultured at the same site, MCMS outbreaks in *P. monodon* do not spread to *P. merguiensis* or *P. japonicus*. Chlorine disinfection is highly effective for destroying the MCMS virus(es), even when entire prawn heads are used in experiments. Surprisingly desiccation (drying prawn heads in the sun) was not effective. Even after one week in the sun there was enough live virus(es) remaining to infect healthy prawns. It is clear the internationally recommended farm clean-up procedures following viral disease on prawn farms will be effective for MCMS. The use of chlorine at 100-200mg/l is recommended for pond disinfection. One of the factors for the decline in MCMS incidence in Queensland is probably due to the implementation of whole-farm destocking and pond disinfection undertaken on many of the affected prawn farms.

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Appendix 1. Intellectual Property

The following was the current status of intellectual property at the time the project ended.

a. Registry of IP known before project commenced:
- 2 kb pair clone of SMV in pGEM vector
- sequence of the first 600-800 base pairs (bp)
- 1st set of PCR primers producing a 260 bp product
- DIG-labelled *in situ* gene probe for SMV

b. Ownership of prior IP:
- The pre-existing IP is all owned by JCU (Agreement – 'Prawn Spawner Mortality Virus Collaborative Agreement 21/12/1995'). However, a proposed distribution of any proceeds is based on funding contributions up to 1995/96, that is 55% James Cook University, 37% Queensland Department of Primary Industries and 8% CSIRO.

c. IP developed to data in project:
- sequence of the whole 2kb insert in the pGEM vector
- 2nd set of PCR primers producing a 200 bp product
- production of a confirmatory dot blot test of 150 bp
- production of a refined *in situ* gene probe (400 bp) which has no vector residues which therefore, should be more specific

d. Comment on IP and how it is currently being handled:
- Contact with patent lawyers has been instigated by JCU to see what is patentable and what is not. Similarly, advice on what should be published in the public domain and what should not be has also occurred. Discussions between the research partners has been instigated.

Appendix 2 – Staff List.

Oonoonba Veterinary Laboratory, Queensland Department of Primary Industries:
IG Anderson, Principal Veterinary Pathologist (Fish Disease)
A Fisk, Senior Technician (Fish Disease)
BF Edgerton, Project Research Scientist (December 1996 – June 1998)
AD Reynolds, Project Research Scientist (July 1998 – September 1999)
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