A National Survey to Verify Freedom from White Spot Syndrome Virus and Yellow Head Virus in Australian Crustaceans

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ABSTRACT

An Australia-wide survey was conducted to determine the infection status of Australian crustaceans for white spot virus (WSSV), yellow head virus (YHV) and gill-associated virus (GAV). The survey was designed using the FreeCalc software package to detect a within-site prevalence of greater than 10% with 95% confidence for each virus. The site prevalence for each virus was assumed to be 10%. Samples of the predominant crustacean species were collected from 66 locations throughout Australia and tested for WSSV. Samples from thirty locations were collected and tested for GAV and YHV. Testing for all viruses involved the use of Polymerase Chain Reaction (PCR) techniques. Neither WSSV nor YHV were detected in any Australian crustaceans. GAV was detected in samples collected from the previously known range of this virus along the Pacific Coast of Queensland and also from Weipa in the Gulf of Carpentaria.

INTRODUCTION

White spot syndrome virus (WSSV) and yellow head virus (YHV) are the major pathogens that affect the prawn farming industry throughout south and southeast Asia. WSSV is a double-stranded DNA virus that is potentially lethal to most of the commercially cultivated penaeid shrimp species and can also cause sub-clinical infections in a range of other crustacaeans including crabs, lobsters and freshwater crayfish (Flegel, 1997). YHV is a single-stranded RNA virus that affects *Penaeus monodon* and has been shown experimentally to infect other penaeid prawns (OIE, 2000). Both WSSV and YHV can cause outbreaks of disease that propogate rapidly and that can result in 100% mortality within a few days.

YHV was first reported in Thailand in 1990 (Limsuwan, 1991), and WSSV first occurred in Chinese Taipei and the Chinese mainland between 1991 and 1992 (Nakano *et al*., 1994). Since that time, both viruses have spread throughout all prawn farming regions of south

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and southeast Asia from Pakistan in the west to Indonesia in the east (OIE, 2001). In 1999, WSSV spread to central and south America. Australia, New Zealand and the islands of the South Pacific are currently free of both WSSV and YHV (OIE, 2001). In 1997, a virus that was morphologically indistinguishable from YHV and that had caused mass mortalities in farmed *Penaeus monodon* was reported from Australia (Spann *et al*., 1997). The virus was named gill-associated virus (GAV) and was subsequently shown to have approximately 85% nucleotide sequence identity with YHV (Cowley *et al*., 1999).

Increasing world trade in agricultural commodities has lead to concern about the potential to introduce exotic diseases through the movement of these commodities. An example of this was the demonstration that green prawns imported into the USA were shown to contain viable WSSV and YHV (Nunan *et al*., 1998). The 1995 outbreak of WSSV and YHV in the United States may have been associated with the inappropriate disposal of waste generated from the processing of imported green prawns (Lightner *et al*., 1997).

The potential threat that imported green commodity prawns poses to Australia was demonstrated when, in November 2000, animals from two aquaculture research facilities in Darwin returned positive PCR tests indicative of WSSV infection. The possible source of infection was traced to imported green commodity prawns used as feed in both establishments. Although no clinical evidence of WSSV was observed, the Consultative Committee on Emergency Animal Diseases (Australia's technical committee for management of the response to emergency disease incidents) considered it prudent, due to the possible presence of viable WSSV in green commodity prawns and the possible diversion of these prawns into the aquaculture feed and bait markets, to conduct a national survey to determine whether WSSV existed in crustacean populations within Australia. Due to the considerable effort involved in collection of the samples, the opportunity was taken to also examine the samples for the presence of YHV and GAV. In this paper, we report on the outcomes of a survey conducted to examine the infection status of Australian crustaceans for WSSV, YHV and GAV.

MATERIALS AND METHODS

A national survey was undertaken to determine whether WSSV, YHV and GAV were present in Australian crustacean populations. The survey design utilised a two-stage sampling strategy as described by Cameron and Baldock (1998). In the absence of a defined sampling frame for wild crustacean populations ("herds") within Australia, two assumptions were made:

1. There are 500, independent, non-interacting populations of crustaceans within Australian coastal waters. This figure was a conservative estimate based on the length of the Australian coastline being 36,735 km (ABS, 1996) and studies on prawn larval advection to estuarine nursery grounds that demonstrated that the limit of recruitment was no more than 65 km from the estuary (Rothlisberg *et al.*, 1996).
2. Sampling at a range of geographically distant sites adequately represented random sampling at the site level of the survey.

Expected prevalence of disease

It was assumed for the purposes of this survey, that if WSSV, YHV or GAV were present in Australia, then it would be present in a minimum of 10% of the crustacean populations

(sites). Within WSSV-infected wild populations of crustaceans in Asia, the prevalence of WSSV varies widely. Typical examples include 6.7% in male Penaeus japonicus from coastal Japan (Wang *et al*., 1998), 26% in P. semisulcatus from the southwest coast of Taiwan (Chen *et al*., 2000), 60% in larvae of Scylla serrata from Taiwan (Hsu *et al*., 1999), and 67% and 74% in P. monodon brooders from Taiwan (Kou *et al*., 1999). Cowley *et al*. (2000) reported that GAV was present in wild populations at a prevalence of greater than 98%. Walker (2000) also reported an extremely high prevalence (> 90%) in wild caught *P. monodon* broodstock from four sites along the North Queensland coast. Little data is available on the prevalence of YHV in wild crustacean populations. The prevalence of YHV in farmed *P. monodon* in the Philippines varied from 13 to 66% (Natividad *et al*., 2002). Based on these published works, it was assumed that the proportion of infected animals within a population would exceed 10%.

Confidence required

The survey was designed to provide a 95% confidence of detecting at least one infected crustacean population within Australia given the assumptions outlined. We also wanted to minimise the chance of wrongly concluding that WSSV or YHV might be present. False positive reactions can occur with any diagnostic test, and these present a particular problem since each reactor must be investigated to determine whether the result is a true positive. Accordingly, a protocol for investigating positive reactions was developed for this survey.

Testing regime

The standard PCR analysis of the Office International des Epizooties (OIE) (OIE, 2000) was completed in one of four separate regional laboratories. Any samples that gave a preliminary positive result in testing were retested at either CSIRO Livestock Industries, Australian Animal Health Laboratory (CSIRO-AAHL) or Long Pocket Laboratories. The survey was designed to allow any samples that tested positive by PCR in both laboratories to be further assessed by bioassay.

Test sensitivity and specificity

None of the PCR tests used in this survey have been validated with field samples to determine the true specificity and sensitivity of the technique. However, validated PCR tests routinely have specificities and sensitivities greater than 95% (Müller-Doblies *et al*., 1998; Peter *et al*., 2000). Although the specificity for the two PCR tests used was not known, the complete test regime of retesting preliminary positive samples and the subsequent use of bioassay if required, was assumed to have an overall specificity of 100%. Based on the known sensitivity of other validated PCR tests, the sensitivity of the PCR testing regime was assumed to be 95%.

Number of samples required

The number of sites to sample and the number of animals per site to sample depend on a range of factors including the expected prevalence of disease (at the site and within site level), the desired confidence level, the sensitivity and specificity of the tests used (Cameron and Baldock, 1998; Garner *et al*., 1997) and the total number of crustacean populations.

The data used to determine sample size is shown in Table 1. Given the values outlined for test specificity, sensitivity and confidence level, a two-stage survey was designed using the Freecalc software program (Cameron and Baldock, 1998). As discussed by Garner *et al*. (1997), there is some flexibility in selecting the number of populations (sites) to sample and the number of individual animals per site, to satisfy the desired confidence level. Taking both convenience and cost into account, it was decided that the survey would require the collection of 30 individuals of the one species at each survey site from a minimum of 30 sites in total. Based on published reports that WSSV is found in a wide range of crustaceans (Lo *et al*., 1996; Otta *et al*., 1999; Wang *et al*., 1998), target species for the survey were the predominant crustacean species in each area.

Table 1. Parameters for sample size calculation.

Site level	**Parameters**
Site sensitivity	0.95
Site specificity	1.00
Number of sites	More than 500
Minimum expected site prevalence	10%
Type 1 error (?)	0.05
Type 2 error (?)	0.05
Confidence	0.95
Power	0.95
Number of sites to test	30
Crustacean level	
Sensitivity	0.95
Specificity	1.00
Population crustaceans at a site	More than 500
Minimum expected site prevalence	10%
Type 1 error (?)	0.05
Type 2 error (?)	0
Confidence	0.95
Power	1.00
Number of crustaceans to test	30
Total number to test	900

Collection of samples

All penaeid prawn samples collected were juvenile or sub-adults as recommended by the OIE (OIE, 2000). Crabs were collected using standard baited pots, and prawns were collected by beam trawl. Some samples were purchased from licenced professional fishermen. Animals were euthanased and either gill tissue (2-3 mm^3) or pleopod immediately placed into preservation medium (ethanol:glycerol:water, 70:20:10) and stored at ambient temperature. Preserved samples were then transported to the testing laboratories.

Extraction of total nucleic acid

Tissue weight was determined. The tissue was gently homogenised in 9.5 vol of CTAB buffer (2% w/v hexadecyl-trimethyl-ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 7.5), 0.25% v/v 2-mercaptoethanol) and incubated to solubilise the TNA. A sample of the homogenate was extracted once with phenol/chloroform/iso-amyl alcohol [24:24:1] and then once with chloroform/iso-amyl alcohol [24:1]. The final aqueous

phase was added to 0.9 vol of 100% iso-propanol and the TNA was precipitated by freezing at ñminus 20°C or below and then collected by centrifugation at 12,000 x g for 15 min at room temperature. The pellet was washed in 70% ethanol, air-dried and then resuspended in sterile distilled water with a volume equivalent to 2X the original tissue weight (i.e. for 50 mg resuspend in 100 µl). This sample was diluted to provide a final concentration of 80 ng/µl TNA for PCR analysis.

Decapod nested-PCR assay

The TNA sample extracted for analysis of WSSV was used for analysis of decapod DNA. Decapod Master Mix, which contains PRIMER SET DP3-2, was overlaid with oil and 0.1 volume of test TNA sample or control sample was added to the tube and mixed by centrifugation immediately before adding to a thermal cycler pre-heated to 80?C. PCR amplification was 1 x (94°C for 2 min.) then 60 x (96°C for 20 s, 55°C for 30 s, 62°C for 20 s, 70°C for 90 s) and finally 1 x (70°C for 5 min., 30°C for 10 min.). After thermal cycling, 10 µl of the PCR reaction mix was removed and examined by agarose gel electrophoresis for the large decapod fragment of 830 bp and for the nested decapod fragment of 240 bp.

Standard WSSV nested-PCR assay

TNA extracts were analysed by the 2-step nested-PCR method of Lo *et al*. (1996, 1997) The sequence of the primers used in PCR analysis is shown in Table 2.

Table 2. Sequence of primers used in PCR analysis of tissues for WSSV.

Primer set	Target	Code	Sequence (5'-3')
WS2	WSSV	(146F1)	ACTACTAACTTCAGCCTATCTAG
	WSSV	(146R1)	TAATGCGGGTGTAATGTTCTTACGA
WS3	WSSV	(146F2)	GTAACTGCCCCTTCCATCTCCA
	WSSV	(146R2)	TACGGCAGCTGCTGCACCTTGT
WS5	WSSV	(1s5)CA	CTCTGGCAGAATCAGACCAGACCCCTGAC
	WSSV	(1a16)	TTCCAGATATCTGGAGAGGAAATTCC
DP3-2	Decapod	(20s2)	CTGCCTTATCA(G/A)CTTTCGAT(G/T)GTAGG
	Decapod	(20a2)	ACTTCCCCCGGAACCCAAAGACT
	Decapod	(20s9)	GGGGGCATTCGTATTGCGA

PCR for degraded WSSV DNA

The following unpublished method for detection of degraded WSSV DNA was developed by Richard Hodgson and Peter Walker of CSIRO Livestock Industries. The master mix, which contains primer set WS5 (see Table 2), was overlaid with oil and 0.1 volume of test

TNA sample or control sample was added to the tube and mixed by centrifugation immediately before adding to a thermal cycler preheated to 85?C. PCR amplification was 1 x (94?C for 2 min) then 60 x (96°C for 20 sec, 55°C for 30 sec, 62°C for 20 sec, 70°C for 90 sec) and finally 1 x (70°C for 5 min, 30°C for 10 min). After thermal cycling, 10µl of the reaction mix was removed and examined by agarose gel electrophoresis for the presence of at least one of the specific WSSV products of 480 bp or the nested products of 420, 385 280 and 198 bp.

Real-Time PCR

Cuticular epithelium from three abdominal segments (or at least one entire gill) was placed in an Eppendorf tube and ground with a disposable plastic pestle. 500 µl of TNET was then added to form a homogeneous mixture, and 12.5 µl of proteinase K (2 mg/ml) and 5 µl of 10% SDS were also added. This mixture was then digested for 1-3 hr at 37°C (with frequent shaking). The digested mixture was extracted with phenol, and DNA was recovered from the subsequent aqueous phase using the "QIAamp Viral RNA Mini Kit" for cell-free extracts (QIAGEN, Valencia, CA, USA) (this kit can also be used for DNA viruses).

The real-time PCR was then set up in a 96-well plate format, with water being used as a negative control, and nucleic acid from the gills of a WSSV experimentally-infected prawn being the positive control. Each control and sample was examined in triplicate. Amplification and analysis of samples was done with the AB 7700 (PE Applied Biosystems) by the method previously described (Dhar *et al*., 1999).

PCR for yellow head virus and gill associated virus

Samples for YHV and GAV detection were processed according to the method of Cowley *et al*. (2002). PCR amplification of YHV specific and GAV specific DNA was conducted by the method of Cowley *et al*. (2002) or by the commercialised version of the same method (IQ2000 test kit, Farming Intelligene Technology, Taiwan) according to the manufacturer's instructions.

RESULTS

In the final survey, a total of 3,081 samples representing 65 batches of at least 30 specimen animals from 56 geographically separate locations throughout Australia were collected and tested for the WSSV. The samples included 51 batches of wild crustacean encompassing the entire Australian coastline (Fig.1) and 17 commercial crustacean farms, hatcheries and research facilities that had populations sourced from the wild or F1 populations originating from broodstock sourced from the wild. For YHV and GAV, a total of 32 batches totalling 1,006 samples from 30 sites were tested.

WSSV Testing

The location of the sites sampled, the species of crustacean sampled, the sample size and the testing results are summarised in Table 3. Of the 65 batches sampled and tested for WSSV infection, 62 sites were negative for WSSV during initial testing whilst preliminary positive results were obtained from three sites. Each positive sample was only positive after the second step of the nested-PCR test, and the results observed were consistent with a level of WSSV close to the lower limit of detection of the test. Duplicate tissues from the

samples that returned preliminary positive results were dispatched to a second independent laboratory for retesting. All duplicate tissues submitted for retesting tested negative with both the Lo PCR and Real Time PCR tests at the second laboratory; therefore, a bioassay was not necessary to resolve the infection status of any samples.

YHV/GAV Testing

The results of testing for YHV and GAV at 30 sites around Australia are summarised in Table 3. All populations sampled were negative for YHV. Seven sites tested positive for GAV. Five of these sites were located on the Pacific coast of Australia north of latitude 27° South and the other two were on the western side of the Gulf of Carpentaria. Each of these findings was consistent with the known distribution of GAV within Australia (Walker, 2000).

Table 3. Location, host species and results of testing various species of crustacean for white spot syndrome virus, yellow head virus and gill-associated virus at sixty-six locations within Australia.

Site No.	State	Site	Species	Sample Size	WSSV Status	YHV Status	GAV Status
1	New South Wales	Botany Bay	Penaeus plebejus	30	negative	negative	negative
2		Manly	*Plagusia chabrus*	35	negative	negative	negative
3		Port Jackson	*Penaeus plebejus/Metapenaeus macleayi*	397	negative	negative	negative
50		Lake Tilba	*Prawn spp.*	30	negative	negative	negative
52		Farm 11	*Penaeus monodon*	270	negative	N.D.2	N.D.
53		Farm 2	*Penaeus monodon*	50	negative	N.D.	N.D.
54		Farm 3	*Penaeus monodon*	50	negative	N.D.	N.D.
55		Farm 4	*Penaeus monodon*	50	negative	N.D.	N.D.
56		Farm 5	*Penaeus monodon*	150	negative	N.D.	N.D.
4	Queen sland	Logan River	*Scylla serrata*	40	negative	N.D.	N.D.
5		Moreton Bay	*Prawn spp.*	37	negative	negative	positive
6		Townsville	*Scylla serrata*	31	negative	negative	negative
7		Townsville	*Penaeus indicus*	57	negative	negative	positive
8		Innisfail	*Penaeus monodon*	53	negative	negative	positive
9		Cairns	*Penaeus monodon*	30	negative	negative	positive
10		Weipa	*Penaeus merguiensis*	30	negative	negative	positive
11		Staaten River	*Scylla serrata*	30	negative	negative	positive
57		Farm 6	*Penaeus monodon*	50	negative	N.D	N.D.
58		Farm 7	*Penaeus monodon*	50	negative	N.D	N.D.
59		Farm 8	*Penaeus monodon*	45	negative	N.D	N.D.
60		Farm 9	*Penaeus monodon*	35	negative	N.D	N.D.
61		Farm 10	*Cherax quadricarinatus*	30	negative	N.D.	N.D.

62		Bribie Island Aquaculture Research Centre	*Scylla serrata*	30	negative	negative	positive
63		Bribie Island Aquaculture Research Centre	*Portunus pelagicus*	35	negative	negative	negative
64		QDPI Research Station	*Cherax quadricarinatus*	30	negative	N.D	N.D
12	Northern Territory	Blackmore River	*Scylla serrata*	30	negative	N.D.	N.D.
13		Elizabeth River	*Carcinus maenas*	30	negative	N.D.	N.D.
14		Nightcliff Jetty	*Carcinus maenas*	30	negative	N.D.	N.D.
15		Rapid Creek	*Scylla serrata*	30	negative	N.D.	N.D.
16		Shoal Bay	*Prawn spp.*	46	negative	N.D.	N.D.
65		Farm 11	*Penaeus monodon*	30	negative	N.D.	N.D.
17	Western Australia	Wyndham – off shore	*Penaeus latisulcatus*	30	negative	negative	negative
18		Wyndham – mud flats	*Penaeus merguiensis*	30	negative	N.D.	N.D.
19		Broome TAFE	*Metopograpsus spp.*	30	negative	N.D.	N.D.
20		Broome	*Penaeus monodon*	30	negative	negative	negative
21		Cable Beach, Broome	*Leptodius spp.*	30	negative	N.D.	N.D.
22		Willy Creek	*Metopograpsus spp.*	30	negative	N.D.	N.D.
23		Exmouth	*Metopograpsus spp.*	30	negative	N.D.	N.D.
24		Exmouth	*Metapenaeus sp.*	30	negative	N.D.	N.D.
25		Exmouth Gulf 1	*Penaeus latisulcatus*	30	negative	N.D.	N.D.
26		Exmouth Gulf 2	*Penaeus monodon*	30	negative	N.D.	N.D.
27		Denham (January)	*Leptodius spp.*	30	negative	N.D.	N.D.
28		Denham (August)	*Leptodius spp.*	30	negative	N.D.	N.D.
29		Geraldton	*Leptograpsus variegates*	30	negative	N.D.	N.D.
30		Fremantle	*Portunus pelagicus*	30	negative	N.D.	N.D.
31		Fremantle	*Penaeus latisulcatus*	30	negative	negative	negative
32		North Cockburn Sound	*Metapenaeus spp.*	30	negative	N.D.	N.D.
33		South Cockburn Sound	*Metapenaeus spp.*	30	negative	N.D.	N.D.
34		Mandurah Estuary	*Penaeus latisulcatus*	30	negative	N.D.	N.D.
35		DeGray River, Port Headland	*Penaeus merguiensis*	30	negative	negative	negative
36		DeGray River, Port Headland	*Penaeus monodon*	30	negative	negative	negative
37		Eagle Hawk Is.	*Metapenaeopsis spp.*	30	negative	negative	negative
38		Nickol Bay,	*Penaeus latisulcatus*	30	negative	negative	negative
39		Solitary Is. Onslow	*Penaeus esculentus*	30	negative	negative	negative
40		Albany	*Portunus pelagicus*	30	negative	N.D.	N.D.

51		Carnarvon	*Metapenaeus endeavouri*	30	N.D.	negative	negative
66		Farm 12	*Penaeus monodon*	30	negative	N.D.	N.D.
67		Farm 13	*Penaeus monodon*	30	negative	N.D.	N.D.
41	Victoria	Altona	*Crab spp.*	30	negative	negative	negative
42		Geelong	*Crab spp.*	30	negative	negative	negative
43		Lakes Entrance	*Crab spp.*	30	negative	negative	negative
48		Portland	*Crab spp.*	30	N.D.	negative	negative
49		Warnambool	*Crab spp.*	30	N.D.	negative	negative
44	Tasmania	Margate Wharf	*Carcinus maenas*	35	negative	negative	negative
45		Kingston	*Crab spp.*	30	negative	negative	negative
68		TAFI Aquaculture	*Jasus verreauxi*	30	negative	negative	negative
46	South Australia	Spencer Gulf	*Penaeus latisulcatus*	110	negative	negative	negative
47		St Vincent's Gulf	*Penaeus latisulcatus*	135	negative	negative	negative
Total				**3170**			

Commercial enterprises are not identified for reasons of commercial confidentiality.
N.D. – Not done – sample was not tested for this virus

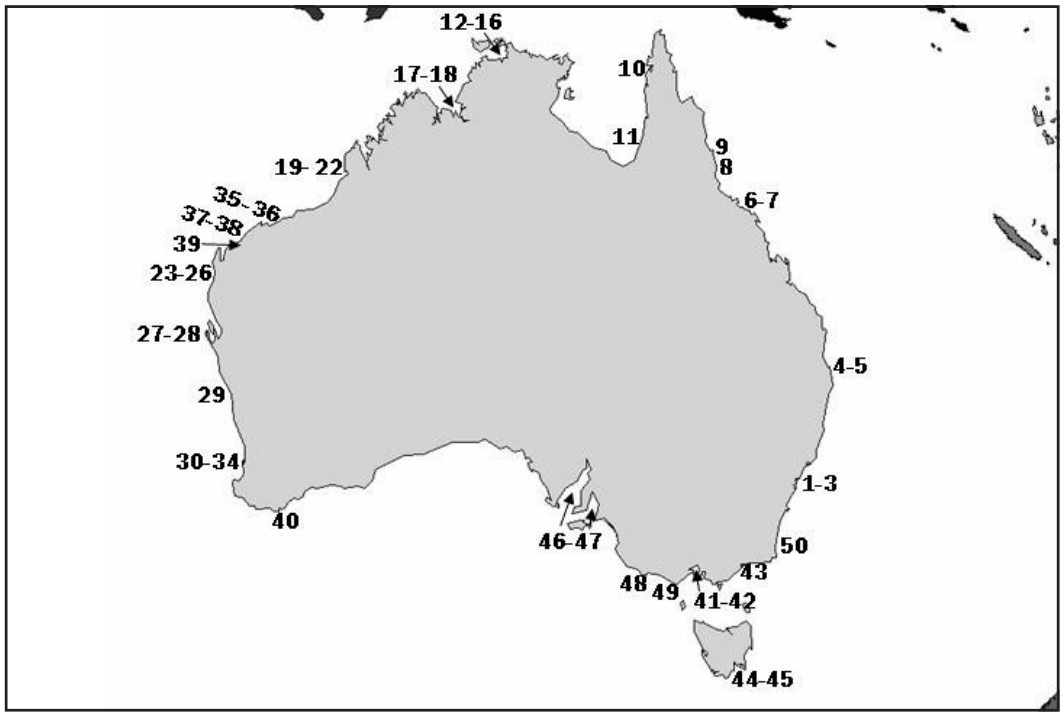

Figure 1. Location of sampling sites for wild crustaceans within Australia. Location numbers correspond to the site numbers listed in Table 3.

DISCUSSION

This study was designed to determine whether Australian crustaceans were infected with WSSV, YHV or GAV. After testing of samples from 3170 crustaceans collected from 59 locations throughout Australia, neither WSSV nor YHV was confirmed in any sample. Thus, our results demonstrate that, at the 95% confidence level, we can conclude that if present, WSSV and YHV occur at an overall prevalence of less than 1.0% (i.e., less than 10% site prevalence and less than 10% within site prevalence). The presence of GAV within its known geographical range along the northern Pacific coastline of Australia was confirmed by our survey. The detection of GAV in *Scylla serrata* extends the known host range of this virus.

The inclusion of 12 commercial *Penaeus monodon* farms in the survey was instructive because, historically, expression of WSSV and YHV as clinical disease has occurred on commercial farms where stocking rates are high and animals are more likely to be stressed. Neither virus was detected on any of the commercial farms, and clinical signs associated with WSSV and YHV have never been observed within Australia. Twelve months prior to the conduct of the current survey, all Australian commercial prawn farms carrying stock were surveyed and found to be free of WSSV (East, unpublished data). In addition to the current survey, an independent survey for WSSV has also been conducted in the area of Darwin Harbour adjacent to the two aquaculture facilities where WSSV PCR-positive samples were detected. That survey has confirmed that WSSV is not present in Darwin Harbour (anon, 2002).

The threat of disease introduction through the use of wild broodstock has lead to the introduction of pre-stocking screening programs in Thailand (Withyachumnarnkul, 1999) and research programs to close the breeding cycle of *P. monodon* in captivity (Preston, 2002). However, until such alternatives are completely effective, wild broodstock with a known disease status are the most effective way of ensuring that disease does not have a major economic impact on the Australian prawn farming industry. Australian crustaceans provide a valuable source of specific pathogen free broodstock for the aquaculture industries of the world. Some producers in Vietnam are now sourcing *P. monodon* broodstock from Western Australia (Brian Jones, pers. comm.), and *P. monodon* post-larvae have been exported to several countries throughout Asia and the Pacific. In conclusion, Australian crustaceans remain free of WSSV and YHV infection, and these viruses remain exotic to Australia.

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