Tactical Research Fund
Aquatic Animal Health Subprogram: Determining the susceptibility of Australian species of prawns to infectious myonecrosis

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Abbreviations

AAHL	Australian Animal Health Laboratory
ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
AP	alkaline phosphatase
APFA	Australian Prawn Farmers Association
CBAD	Centre for Brackishwater Aquaculture Development
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C_T	threshold cycle
DIG	digoxigenin
Dpi	days post-inoculation
FRDC	Fisheries Research and Development Corporation
IHHNV	Infectious hypodermal and haematopoietic necrosis virus
i.m.	intramuscular
IMN	infectious myonecrosis
IMNV	infectious myonecrosis virus
ISH	*In situ* hybridisation
MBSIA	Moreton Bay Seafood Industry Association
OIE	Office International des Epizooties – the World Organisation for Animal Health
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
RT-qPCR	reverse transcriptase real-time polymerase chain reaction
TS	Taura syndrome
TSV	Taura syndrome virus
WSD	whitespot disease
WSV	whitespot syndrome virus
YHV	yellowhead virus

Executive Summary

What the report is about

Scientists at the CSIRO Australian Animal Health Laboratory (AAHL) in Geelong Victoria, with assistance from Indonesian scientists at the Centre for Brackishwater Aquaculture Development (CBAD), Jepara, Indonesia have demonstrated that two prawn species of commercial importance to Australia are susceptible to the exotic virus, infectious myonecrosis virus (IMNV). IMNV causes infectious myonecrosis, a disease of penaeid prawns which has been reported to occur in north-eastern Brazil, in the East Java Island, west Java, Sumatra, Bangka, west Borneo, south Sulawesi, Bali, Lombok and Sumbawa in South-East Asia and possibly in other South-East Asian countries (OIE, 2014).

IMNV is known to cause significant disease outbreaks, associated with mortalities, in farmed Pacific white shrimp (*Litopenaeus vannamei*) i.e. by natural infection. In addition, the Pacific blue shrimp (*Penaeus stylirostris*) and the black tiger shrimp (*P. monodon*) are susceptible to experimental infection with IMNV (OIE, 2014). Apart from these data there is no information on susceptibility of other prawn species.

In 2011-12, Australian commercial prawn production was valued at $265 million (ABARES, 2013) and, together, commercial and non-commercial prawns are a significant resource of which the farmed banana prawn (*Fenneropenaeus merguiensis*) and the wild brown tiger prawn (*Penaeus esculentus*) are important species. It is important to know whether prawn species such as these are susceptible to infection by IMNV to assist in determining the risk this exotic virus may pose should there be an incursion.

Thus, in collaboration with MCBAD Indonesia, infectivity trials were undertaken (1) at AAHL to determine the susceptibility of IMNV to the banana prawn and the brown tiger prawn, and (2) at MCBAD, using the natural host the Pacific white shrimp as positive control.

Background

The prawn fishery, including prawn aquaculture, is an important natural resource for Australia that is also the basis for a valuable export industry. Fortunately, the Australian prawn industry is free from many of the diseases that have devastated prawn aquaculture overseas at one time or another, e.g., the estimated impact of white spot disease (WSD), caused by white spot syndrome virus (WSV) in Asia alone after its emergence in 1992 until 2001, was US$4-6 billion (Lightner, 2003). In the Americas, the emergence of WSD in 1999 resulted in immediate losses estimated at US$1 billion to 2001.

Infectious myonecrosis (IMN) is a viral disease that has caused significant disease outbreaks and mortalities in farmed *Litopenaeus vannamei* (Pacific white shrimp) overseas (OIE, 2014). The economic loss in Brazil alone was estimated to be US$20 million in 2003 (Tang et al., 2005). While *L. vannamei* is considered the principal (natural) host, experimental infection of *Penaeus stylirostris* (Pacific blue shrimp) and *P. monodon* (black tiger shrimp) has been reported (Tang et al., 2005). The susceptibility of other shrimp/prawn species is unknown. Information on the susceptibility of prawn species important to Australia is lacking. Using the bio-secure containment facility provided by the CSIRO Australian Animal Health Laboratory, this study provides significant new information on the susceptibility of two commercially important species of Australian prawns, *F. merguiensis* (banana prawn) and *P. esculentus* (brown tiger prawn), following exposure to exotic IMNV. Such information is important to policy-makers, regulators and primary producers with respect to relevant biosecurity issues at all levels of government.

Aims/objectives

1. Import infectious myonecrosis virus (IMNV) of known pathogenicity
2. Determine the susceptibility of banana prawns to IMNV
3. Determine the susceptibility of brown tiger prawns to IMNV

Methodology

An infectious inoculum of IMNV was prepared at MCBAD, Jepara, Indonesia and transferred to CSIRO AAHL, Geelong. At Geelong, the inoculum was inoculated (i.m.) into banana prawns and brown tiger prawns which were subsequently monitored for signs of infection and disease. The prawns were sampled on a daily basis post-inoculation and tissues were processed for determining the presence of IMNV infection and disease using OIE methods. Following this first trial a second series of experiments were conducted to simulate natural modes of viral transmission and confirm susceptibility according to criteria developed by the OIE (OIE, 2014a).

Results/key findings

This investigation has demonstrated that the two commercial species of prawns of Australian origin, *Fenneropenaeus merguiensis* and *Penaeus esculentus*, are susceptible to infection with the exotic virus IMNV. Such information is important to policy-makers, regulators and primary producers with respect to relevant biosecurity issues at all levels of government.

Implications for relevant stakeholders

While this project was limited to investigating the susceptibility of two important prawn species, the results suggest that the host range for IMNV is broader than previous data had indicated.

Recommendations

It is recommended that industry, regulators at all levels of government and the prawn health community in general note the results of this project and their implications with respect to biosecurity.

Keywords

Infectious myonecrosis (IMN); infectious myonecrosis virus (IMNV); banana prawn (*Fenneropenaeus merguiensis*); brown tiger prawn (*Penaeus esculentus*); *in vivo* infectivity trials; susceptibility; prawn virus

Introduction

Australian prawn production (2011-12) at around 22.5 kilotonnes is valued in excess of AU$265 million (ABARES, 2013). Clearly, the prawn fishery is an important natural resource for Australia that is also the basis for a valuable export industry. In addition, prawn aquaculture is a significant industry in northern Australia and accounts for around 15% (4 kilotonnes with a value of approx. AU$60 million) of the total volume of Australian prawn production. Fortunately, the Australian prawn industry is free from many of the diseases that have devastated prawn production overseas at one time or another. For example, it has been estimated that the impact of white spot disease (WSD), caused by white spot syndrome virus (WSV) in Asia alone after its emergence in 1992 until 2001, was US$4-6 billion (Lightner, 2003). In the Americas, the emergence of WSD in 1999 resulted in immediate losses estimated at US$1 billion to 2001. The combined impacts of Taura syndrome virus (TSV) and infectious hypodermal and haematopoietic necrosis (IHHNV), in the Americas has been estimated at US$1.5-3 billion (Hasson et al., 1999; Lightner 2003), the consequences of disease emergence in some countries being so severe that shrimp production has never fully recovered.

Infectious myonecrosis (IMN) is a viral disease that has caused significant disease outbreaks and mortalities in farmed *Litopenaeus vannamei* (Pacific white shrimp) in Brazil and South-East Asia, including Indonesia (OIE, 2014). The economic loss in Brazil was estimated to be US$20 million in 2003 (Tang et al., 2005). While *L. vannamei* is considered the principal (natural) host, experimental infection of *Penaeus stylirostris* (Pacific blue shrimp) and *P. monodon* (black tiger shrimp) has been reported (Tang et al., 2005). The susceptibility of other shrimp/prawn species is unknown. Information on the susceptibility of prawn species important to Australia, including banana prawns (*Fenneropenaeus merguiensis*) and brown tiger prawns (*P. esculentus*) is lacking. In 2010, the General Manager SEAFARM, approached CSIRO-AAHL with concerns about IMN caused by infectious myonecrosis virus (IMNV) that is exotic to Australia. Specifically, information on the host range for this virus was requested. While it is known that *L. vannamei* (which does not occur in Australia) is highly susceptible to IMNV and *P. monodon* (commonly farmed both overseas and in Australia) can be infected experimentally with the virus, the susceptibility of other prawn species occurring in Australia such as *F. merguiensis* (banana prawn), *P. esculentus* (brown tiger prawn) is not known. The Australian Prawn Farmers Association (APFA), in general, had similar concerns. A potential project was discussed with FRDC and it was indicated that a project which included a wild species such as brown tiger prawn (*P. esculentus*), as well as the farmed species, banana prawns (*F. merguiensis* which would be provided by SEAFARM), would be considered and was approved for funding. Assistance was sought from the seafood industry in sourcing brown tiger prawns and the Moreton Bay Seafood Industry Association (MBSIA) agreed to collaborate by organising collection of wild-caught brown tiger prawns for the project. This project would provide information on the susceptibility of two important species of Australian prawns to exotic IMNV. Such information is important to policy-makers, regulators and primary producers with respect to relevant biosecurity issues at all levels of government.

Objectives

1. Import infectious myonecrosis virus (IMNV) of known pathogenicity
2. Determine the susceptibility of banana prawns to IMNV
3. Determine the susceptibility of brown tiger prawns to IMNV

Methodology

1. Production of IMNV of known pathogenicity

Since 2004, when infectious myonecrosis was first recognised in farmed *L. vannamei* in Brazil, it is thought to have spread to Indonesia through the introduction of contaminated seed stock (Senapin *et al.* 2007). The virus strains from Brazil and Indonesia are both known to cause severe disease in *L. vannamei* which is presumed to be the natural host. These strains have been characterised and compared, and share 99.6% similarity at the nucleotide level over the full 7.5 kb genome (Senapin *et al.* 2007). On this basis, and given the geographic proximity of reported outbreaks in Java and the surrounding archipelago, it was decided to use an Asian isolate of IMNV to determine the susceptibility of the Australian prawns to IMNV infection. Although there have been widespread reports of disease associated with IMNV in South-East Asia, the only confirmed infections of IMNV are from Indonesia (Senapin *et al.* 2011).

As a result of previous collaborative projects between CSIRO and the Indonesian Agencies for Aquaculture and Fisheries Research, well-established links are in place with government laboratories in Indonesia with access to both susceptible prawn species (*Litopenaeus vannamei*) and infectious IMN virus from recent disease outbreaks. Despite reports of major disease outbreaks in commercial aquaculture facilities in the 12 months preceding commencement of the project, the project was delayed because our principal collaborating partner in Indonesia was on extended leave and there was difficulty finding a source of infectious virus stored in the laboratory.

Current methods for *in vitro* propagation of prawn viruses are unreliable; therefore preparation of infectious virus for use in experimental challenges was undertaken in susceptible shrimp. In this case, the principal natural host of IMNV, the Pacific white shrimp (*L. vannamei*), is exotic to Australia, so the procedures required to propagate the virus and confirm infectivity of the inoculum were carried out in Indonesia at the Fish Health and Environment Laboratory, Main Centre for Brackishwater Aquaculture Development (MCBAD), Jepara, Central Java, Indonesia. IMNV is enzootic in this area.

Two attempts were made to prepare stocks of infectious virus for the susceptibility challenge. A stock of infectious IMNV haemolymph (identity confirmed locally in Indonesia by OIE-recommended RT-PCR test) was used to infect a small group of *L. vannamei* (n = 10) by parenteral injection. The pooled haemolymph and abdominal muscle from these animals was used to infect a further 2 groups of *L. vannamei,* one by injection and the other by *ad libitum* feeding of infected muscle tissues. Individual fixed samples of haemolymph and muscle from these animals were transferred to Australia for confirmatory testing at CSIRO AAHL (12:02305). While the samples from the injected group contained sufficient IMNV for use in challenge (mean C_T of IMNV OIE real-time PCR = 29.26) the animals infected by oral administration had much lower levels of virus (mean C_T of IMNV OIE real-time PCR = 40.23) with marked variation in the levels of virus in individual animals. This is consistent with the observation that infection by feeding is less predictable than injection for infecting prawns with IMNV. Further testing of these samples revealed that the group infected by injection were co-infected with TSV (mean real-time PCR C_T = 30.57). On this basis these infectious tissues, still stored in Indonesia, were rejected for use in this study.

A second experiment to establish stocks of infectious IMNV for challenge was carried out in December 2012. Given recent work at that time that suggested infection by feeding could be unreliable and that the concentration of IMNV in haemolymph (up to 5.08×10^8 copies/µg of total RNA) was higher than pleopods or gills (Cabral da Silva *et al.,* 2011), it was decided to establish a stock of infectious IMNV haemolymph only. Apart from containing high levels of virus, haemolymph provides a homogeneous sample which can be diluted accurately and reproducibly when preparing inocula and samples for assay.

Clinically normal *L. vannamei (*mean weight 17.5 g and length 13.6 cm) were obtained from a commercial grower in Situbondo, East Java. The shrimp were transferred to MCBAD and held in 25‰ sea water pre-treated with calcium hypochlorite. The 20 animals in each test group were divided between three 20 L tanks and allowed to acclimatise at 26.5°C prior to inoculation. Three groups of 20 animals were injected (i.m.) into the 2nd abdominal segment, with 50µl of a 1:40 dilution of IMNV haemolymph provided by MCBAD. The virus for group 1 originated from Banyuwangi, East Java; the virus from group 2 came from

Kendal, Central Java; and the virus for group 3 originated from *P. monodon* brood stock at MCBAD thought to have a low-level persistent infection of IMNV. In each case a sterile saline solution (25‰) was used as diluent. A further 2 groups of 10 animals were used as negative controls, all animals being maintained under the same conditions. Control group A was inoculated with 50 µl of the sterile saline diluent and control group B received no treatment. In contrast to all the groups inoculated, the animals from IMNV group 1 reacted badly to the first inoculation and died within 12 hours. This was thought to be a toxic reaction and this group was immediately replaced with a further 20 animals inoculated using the same haemolymph as before (Banyuwangi) but at a higher dilution of 1:80. No further mortalities were observed in any group.

Animals previously infected with IMNV using the above procedure at MCBAD typically produced gross lesions within 5 days post infection, with haemolymph and muscle testing strongly positive by the OIE-recommended one-step conventional PCR. Although all the animals in this experiment appeared normal, with the exception of 1 animal in IMNV group 1 which displayed opaque muscle at the site of inoculation (Figure 1), all the animals were euthanized 5 days post-infection. Individual haemolymph samples were taken from the ventral sinus using a 1 ml tuberculin syringe and 25G hypodermic needle, using a small amount (<5% of the total volume) of 10% w/v sodium citrate as anticoagulant. The individual samples from each group were pooled, and passed through a 0.2 µm membrane filter (Sarstedt) prior to dispensing into 2 ml NUNC cryovials. In order to maintain viral infectivity, individual vials were immediately snap frozen on dry-ice at the MCBAD laboratory and kept frozen at all times thereafter. A range of other tissues were also taken and fixed in 80% ethanol and Davidson's fixative for confirmatory testing at CSIRO AAHL.

Preliminary screening of the 3 candidate haemolymph preparations using the OIE conventional RT-PCR at MCBAD showed that group 3 was either uninfected or had levels of virus below the detection level of the PCR and on this basis these samples were discarded. The following fixed samples, with corresponding frozen samples stored at -80°C, were collected for detailed characterisation and analysis prior to shipment of the virus to the microbiologically secure facility at CSIRO AAHL. These samples also provided a source of viral RNA used to prepare PCR controls for assessment of the IMNV inoculum and for the preparation of a DIG-probe for ISH and assessment of viral replication in Australian shrimp, if required.

1. Untreated shrimp: 10 tubes (gill, pleopod and muscle, 80% ethanol-fixed)
2. Injection (1st group): 10 tubes (gill, pleopod and muscle, 80% ethanol-fixed)
3. Injection (1st group): 1 tube (haemolymph (pool); 80% ethanol-fixed)
4. Injection (2nd group): 20 tubes (gill, pleopod and muscle, 80% ethanol-fixed)
5. Injection (2nd group): 2 tubes (haemolymph (pool), 80% ethanol-fixed)
6. 1 tube (3 mm slice of muscle for histology, 70% ethanol-fixed)

2. Importation of IMNV of known pathogenicity

The infectious stocks of IMN virus for use in susceptibility challenge experiments were imported in the form of pooled haemolymph (Group 1: 12:03191-1 and Group 2: 12:03191-2) collected from infected *L. vannamei*. A commercial air-courier was used to transport the samples to Australia. All samples were imported with the approval of AQIS subject to import permit IP10020358. On arrival at CSIRO-AAHL, Geelong the infectious samples were immediately transferred to low-temperature storage in the biologically secure containment area designated for work with exotic animal pathogens. All subsequent work with these samples, including PCR analysis, sequencing and animal inoculation was confined to the "secure area".

3. Preparation of challenge inoculum

The OIE recommended real-time (RT-qPCR) and conventional RT-PCR for IMNV (OIE, 2014) were used to confirm the presence of IMNV in haemolymph samples. All PCR amplification products from conventional PCR were confirmed by DNA sequencing. The haemolymph samples were further tested by PCR to confirm the absence of other serious viral pathogens, including whitespot virus (WSV), yellowhead virus (YHV), infectious hypodermal and haematopoietic necrosis (IHHNV) and Taura syndrome virus (TSV).

Testing of the candidate haemolymph preparations for pathogenicity and susceptibility trials showed that of the 3 potentially infectious pools the haemolymph from Group 1 "High" (12:3198-1) provided the highest levels of IMNV with a mean C_T of 18.23.

Given the high levels of virus indicated by real-time PCR, it was decided to further reduce the possible adverse effects of inoculating neat haemolymph into prawns encountered in Indonesia by diluting the haemolymph 1:100. All subsequent IMNV inoculation of the target Australian species and the natural host in Indonesia was carried out at this dilution.

4. Challenge of the natural host and Australian native species

Samples of frozen haemolymph corresponding to those transferred to the bio-secure facilities at CSIRO-AAHL were also maintained at -80°C in Indonesia at the MCBAD laboratory. In this way all inocula for subsequent challenge experiments in both Australia and Indonesia were derived from the same source and could be prepared and administered according to the procedures developed using the natural host in Indonesia.

4.1. Experimental Challenge 1 – Intramuscular Injection

In Australia, *F. merguiensis* (banana prawn; mean weight 21.1 gram) obtained from Seafarm and *P. esculentus* (brown tiger prawns; mean weight 28.7 gram) obtained from MBSIA were transferred from Queensland to Geelong, housed in the AAHL bio-secure aquarium facility and allowed to acclimatise in 100 µm-filtered seawater, obtained from Great Southern Waters, Indented Head, Victoria, for 4-6 days prior to infection. Water was changed (50% v/v) on every other day. Water temperature was maintained at 24-25°C. Each of the experimental groups (negative controls, IMNV-inoculated) was held in separate isolation rooms to prevent cross-contamination. Replicate tanks containing 5-20 prawns per 100 L sea-water, were set up for each treatment group and all treatments were performed in duplicate. Replicate tanks of adult prawns were challenged by intramuscular (i.m.) injection with 100 µl of a 1:100 dilution of pooled IMNV haemolymph (see Table 1 for experimental set-up). Clearly, intramuscular injection is not a natural route of infection but was considered useful for these experimental infections as the test animals could not be held for an extended period of time and in order to demonstrate any trend of increasing levels of virus (replication) it was necessary to ensure that individual animals did receive a uniform dose between replicate groups.

In addition, the transit time from Queensland to Geelong for the farmed banana prawns was greater than 24 hours and approximately 50% mortality occurred thus limiting the number of prawns available for experimentation.

Table 1. Summary of experimental set-up for inoculation study

Tank No.	Experimental Group	Prawn species	Number of prawns	Samples taken
1	Uninfected	Banana	5	Days 0 and 10 for histology, ISH and PCR analysis
2	Uninfected	Banana	5	Days 0 and 10 for histology, ISH and PCR analysis
3	Uninfected	Brown tiger	10	Days 0 and 10 for histology, ISH and PCR analysis
4	Uninfected	Brown tiger	10	Days 0 and 10 for histology, ISH and PCR analysis
5	Infected	Banana	12	1 animal per day, from d1 to d10 post infection – PCR, histology and ISH. HL taken and stored from d5.
6	Infected	Banana	13	1 animal per day, from d1 to d10 post infection – PCR, histology and ISH. HL taken and stored from d5.
7	Infected	Brown tiger	14	4 animals per day, from d1 to d10 post infection – PCR, histology and ISH. HL taken and stored from d5
8	Infected	Brown tiger	15	
9	Infected	Brown tiger	14	
10	Infected	Brown tiger	15	None – to be used for cumulative mortality
11	Infected	Brown tiger	15	None – to be used for cumulative mortality

Prawns were monitored daily for clinical signs. Samples were taken for laboratory analysis including histopathology, *in situ* hybridisation (ISH) and PCR analysis using standard procedures. In addition, any moribund prawns, or prawns with abnormal clinical signs (cessation of feeding; lethargy), were euthanized and samples (haemolymph, gills, head/thorax and tail) were collected to confirm (or not) replication of virus and to assess the degree of pathology in Australian prawn species. A syringe and hypodermic needle with 10% sodium citrate as anti-coagulant, was used to collect haemolymph from the major ventral sinus of each live prawn. A small volume of haemolymph (100 µl) was set aside for PCR with the remaining sample being stored at -80°C as a future source of live passaged virus. Samples of abdominal muscle were fixed in 70% (v/v) ethanol for PCR and Davidson's AFA fixative for histology and ISH. Davidson's fixed samples were transferred to 70% (v/v) ethanol after 24 hours, pending routine processing for histology and ISH (OIE, 2014).

4.2. Trial 2 – Water Borne Exposure and Exposure by Feeding of Infected Tissue

In addition to the experiments to determine if IMNV virus was able to replicate in test species following injection, previously used to determine IMNV susceptibility in other prawn species (Tang *et al*., 2005), additional experimental infections were carried out to establish host susceptibility using criteria developed by the OIE (OIE, 2014a).

In the absence of specific procedures for experimental exposure, OIE guidelines require that susceptibility should be assessed according to the following 3 criteria.

1. Identification of the causative agent must have been conducted in accordance with methods described in the disease chapters in the *Aquatic Manual*. As before, all screening for IMNV was based on the methods outlined in Chapter 2.2.3 of the *Aquatic Manual* (OIE, 2014).
2. Infection should be demonstrated by data on natural occurrence, data from non-invasive experimental procedures (e.g. cohabitation, predation, or – when relevant – via intermediate hosts or vectors), or data from invasive experimental procedures that mimic the natural route of infection.

Despite the limitations imposed using limited animal stocks held under laboratory conditions the second round of experiments included 3 challenge methods to simulate natural routes of infection including water-borne exposure and exposure by feeding.

3. A combination of criteria should be used to assess infection of a host species. These include i) presence of an infectious or a viable organism, in or on, the live aquatic animal; ii) evidence of multiplication or other development of the organism; iii) clinical and pathological changes associated with the infection; iv) specific location of the pathogen.

All the procedures in the second series of challenge experiments were conducted using the same sources of farmed banana prawn (*F. merguiensis*) and wild-caught brown tiger prawns (*P. esculentus*) transferred from Queensland to the laboratory in Geelong, as described previously. The same pool of infectious haemolymph that was previously used to demonstrate virus replication in the target species provided the source of IMNV. In order to allow for equipment or other failure in test units, all treatments were carried out using duplicate test groups for each procedure. The same water temperature, salinities and water management procedures were also maintained. To reduce the amount of IMNV haemolymph used, only one group of each test species was used for direct bath exposure. For the purpose of sequential sampling over the time course of the experiments duplicate test units were regarded as a single group and sampling was divided equally between all animals subjected to the same treatment.

(1) Exposure by Co-habitation with IMNV Infected Animals

Twenty-two individuals of each species (*F. merguiensis* and *P. esculentus*), each consisting of 2 tanks of 11 animals for each species, were used for co-habitation of experimentally infected animals with naïve test animals. This co-habitation was intended as a non-invasive procedure to assess susceptibility of the test species by water-borne transmission of virus.

Each test tank contained 5 animals infected with IMNV by parenteral inoculation as described. The infected animals were held inside a floating cage to prevent direct contact or cannibalism as the health of inoculated animals deteriorated. The inoculated and test animals were maintained for 20

days following initial inoculation. Two test animals were sampled for histological examination and molecular analysis every second day starting 2 days post-exposure (n=22 animals for each test species). All prawns inoculated by injection were tested to confirm *in vivo* virus replication by RT-qPCR. In order to confirm release of IMNV from infected animals into the surrounding water, a 30 ml water sample was taken from each tank at days 12, 16 and 20 and stored at -80°C. The water samples were later concentrated by ultracentrifugation (100,000 X *g* for 3 hours) and the pellets extracted for IMNV RT-qPCR analysis.

(2) Exposure by Feeding of Infected Tissues

Twenty individuals of each species (*F. merguiensis* and *P. esculentus*), consisting of 2 tanks of 10 animals for each species, were exposed to IMNV by feeding of infected tissues. This was intended as a non-invasive procedure to assess susceptibility with transmission by simulated predation of infected animals.

In contrast to the first challenge trial, where the brown tiger prawns appeared to be in better condition than the banana prawns following transport, the banana prawns (*F. merguiensis*) for the second series of experiments appeared less stressed and were used to prepare infected tissues for feeding. Twenty-two banana prawns were inoculated by intramuscular injection as described. Previous analysis by RT-qPCR showed levels of IMNV peaked around 7 to 8 days post-inoculation. On this basis animals for feeding were maintained for 7 days to allow virus replication, after which they were euthanized and individually screened by OIE RT-qPCR to confirm the presence of IMNV. The infected abdominal segments with pleopods attached were removed and maintained at 4°C and fed to naive test animals in equal proportions over the following 3 days after which the normal feeding of dried pellets was resumed. Test animals were maintained for 12 days following the first feeding of infected tissue. Two test animals were sampled each day starting 3 days post-exposure (n=20 for each test species), where corresponding samples of abdominal muscle and attached pleopods were fixed in ethanol and in Davidson's AFA fixative for molecular analysis and histological examination, respectively.

(3) Bath Exposure

As it was uncertain when and if IMNV would be released into the water over the course of the co-habitation procedure, this bath experiment was intended as a non-invasive procedure to assess susceptibility of the test species by water-borne exposure to the virus. Five animals of each species were exposed directly to infectious IMNV haemolymph diluted with seawater.

Haemolymph was diluted 1:10 with seawater and approximately 2 ml dripped directly onto the ventral surface of each test animal to ensure contact. The "run-off" of the 1:10 inoculum was collected and diluted with seawater to provide sufficient volume to submerge the test animals. In this case the final dilution of haemolymph was 1:2000 in a volume of 2 litres. Test animals were left for 3 hours after which both the prawns and the inoculum were transferred to their respective holding tanks. The animals were maintained for 20 days before sampling for histology and molecular analysis.

(4) Control animals

Fixed samples for molecular analysis were collected from 3 animals of each test species at day 0, after animals had acclimatised but before any infectious materials were used. A further 5 individuals of each species were maintained under the same conditions as the test animals in a separate isolation room as a source of negative controls. These animals were maintained on dry pellets, but were also fed uninfected prawn tissue *per os* over 3 days (days 8 to 10).

5. Sequence Confirmation of IMNV

Selected RNA samples corresponding to the animals with the highest C_T values from each of the treatment groups and the water sampled from the co-habitation tanks were amplified by OIE IMNV conventional RT-nPCR, for sequence confirmation. PCR products were excised and purified by standard methods (QIAGEN QIAquick Gel Extraction Kit Cat. No. 28706) and used as a source of template for DNA sequencing using the corresponding OIE primers.

6. Assessment of Susceptibility of Australian Prawn Species to IMNV Infection

The susceptibility of Australian prawn species to IMNV was established relative to the morbidity and survival of the natural host (*L. vannamei*) in the virus challenge experiments. In addition, the degree of cellular pathology was assessed by histopathology and was compared to the distribution and localisation of virus demonstrated by ISH.

7. Polymerase chain reaction

At AAHL, both the OIE conventional nested RT-PCR (RT-nPCR) and the real-time RT-PCR (RT-qPCR) were used to confirm and quantify the presence of IMNV in prawn tissues. All testing was carried out as described in the OIE Manual of Diagnostic Methods for Aquatic Animals (OIE, 2014). Real-time PCR testing was performed in triplicate using Taqman Fast Virus 1-Step Master Mix (Life Technologies Cat: 4444434), for conventional PCR, individual tests were performed using the SuperScript III One-Step RT-PCR system with Platinum Taq (Life Technologies Cat: 12574-026) and Qiagen HotStarTaq Master Mix (Qiagen Cat: 203445) for second-step amplification.

To facilitate the comparison of IMNV levels from individual samples over the course of the susceptibility challenge, extractions of RNA were standardised so that the amount of starting material for each sample from each tissue type was the same. RNA was extracted from 100 µl of haemolymph and, for abdominal muscle, small pieces of representative tissues, approximately 100 mg, were collected, weighed, and homogenised by bead-beating in AVL buffer (Qiagen Cat: 19073) prior to nucleic acid extraction from 50 µl of supernatant using the MagMAX Express 96 system (Life Technologies). All samples of haemolymph and muscle were processed at the one time, as a single batch using the same reagents. In each case the nucleic acid was eluted in a final volume of 50 µl.

Prior to any PCR analysis, nucleic acids derived from both haemolymph and muscle were first screened using the Decapod-specific conventional PCR primers 143F/145 from the OIE Manual of Diagnostic Methods for Aquatic Animals, Chapter 2.2.6 White spot disease (OIE, 2014b) to confirm nucleic acid extraction and the absence of PCR inhibition (results not shown). While DNA sequencing was used to confirm IMNV in the inoculum, not all conventional PCR products from the challenge were confirmed by sequencing. In this case, bands from 5 representative samples were excised and sequenced to confirm identity.

At MCBAD, RNA isolation was performed using the QIAGEN RNeasy Mini Kit (Qiagen Cat: 74104). Real-time PCR facilities were not available at the time this work was conducted and so the OIE conventional RT-PCR (OIE, 2014) was used to confirm presence of IMNV in prawn tissues.

8. *In situ* hybridisation

The IMNV *in situ* hybridization (ISH) probe was prepared and used as described in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2014). A sample of cDNA was prepared from the IMNV challenge strain, using haemolymph as a source of RNA (Invitrogen Superscript III First Strand Synthesis Supermix kit Cat: 18080-400). The DIG-labelled probe was made using the Roche PCR DIG Probe Synthesis kit (Cat: 11636090910) using 40 cycles of amplification with denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 1 minute.

In situ hybridisation was carried out using standard procedures. Briefly, 4mm sections of paraffin-embedded tissue were placed on commercially coated ISH slides (Dako) and allowed to dry. Sections were incubated at 65^{o}C for 45 min, deparaffinized, and then rehydrated to distilled water. Sections were covered with 200µl 100µg/ml proteinase K in TNE buffer and incubated at 37^{o}C for 30 min. Slides were washed in cold 4% formaldehyde for 5 min, then in 2x SSC solution at ambient room temperature for 30 min.

Sections were pre-hybridized in 200µl hybridization buffer for 30 min at 42^{o}C in a hybridization chamber. Sections were overlaid with 200µl hybridization solution containing IMNV probe (approx. 1µg/ml), covered with a coverslip, and then heated at 85^{o}C for 10 min. Slides were quenched on ice for 5 min, and then incubated overnight at 42^{o}C in a hybridization chamber.

Coverslips were removed and the slides were washed in 2x SSC (2 x 15 min), 1x SSC (2 x 5 min), 0.5x SSC (2 x 5 min) and briefly in Tris-NaCl buffer. Sections were overlaid with 200µl blocking solution (0.5% skim milk

in Tris-NaCl buffer) and incubated at 37^{o}C for 30 min. Sections were covered with 200µl sheep anti-DIG-AP antibody diluted 1/100 in blocking solution and incubated at 37^{o}C for 30 min.

Slides were washed in Tris-NaCl buffer, and then equilibrated in Tris-NaCl-$MgCl_2$ buffer. Sections were overlaid with 500µl development solution (NBT/BCIP) and incubated in a humid container in the dark at room temperature.

Slides were monitored for color development by periodic checking under a light microscope, and the reaction was stopped after 1.5 hours by immersing slides in distilled water. Slides were counterstained for 30 seconds with 0.5% Bismarck brown, and then mounted with permanent mounting medium and cover-slip.

Sections were examined using light microscopy. Lesions with specific dark blue-black staining indicated presence of IMNV.

Results & Discussion

1. Characterisation of IMNV inoculum

The haemolymph dilution used as inoculum in the challenge experiments was subject to the need to avoid toxic effects in test animals while at the same time providing sufficient virus to allow infection and possible virus replication in susceptible animals. While normally a 1:40 dilution of haemolymph results in successful inoculation (Dr Handayani, pers. comm.) our experience was that for one group of shrimp inoculated in Indonesia a 1:80 dilution was required to reduce toxic effects and avoid post-inoculation shock and mortality. Given the size of test animals and the high levels of virus in the haemolymph a slightly higher volume and dilution (100 µl of 1:100 IMNV 12:03198-1) was used for the susceptibility challenge. The infectivity and pathogenicity of this IMNV inoculum was confirmed by parallel inoculation of the natural host *L. vannamei* at the MCBAD in Indonesia, using the same stock at the same dilution used at CSIRO AAHL.

Prior to challenge, the inoculum was analysed by qPCR for the presence of adventitious agents such as YHV, TSV, WSV, IHHNV and IMNV. Results (Table 2) demonstrated that the inoculum contained significant levels of IMNV (C_T=24.56) and was free of YHV (C_T>45.00), TSV (C_T>45.00), WSV (C_T>45.00) and IHHNV (C_T=42.18).

Table 2. PCR analysis of the inoculum used in the experimental infections

SAMPLE	YHV	TSV	WSSV	IHHNV	IMNV
1/100 haemolymph	>45.00	>45.00	>45.00	42.18[a]	24.56
No template control	>45.00	>45.00	>45.00	>45.00	>45.00
Positive control	32.8	20.16	28.65	22.11	16.59

[a]While the qPCR result for IHHNV might indicate the presence of very low levels of this virus, the amplification curves for this test were not typical when compared to IHHNV positive control.

2. Determination of the susceptibility of banana prawns to IMNV

2.1. Experimental Challenge 1 – Intramuscular Injection

2.1.1. Clinical signs of disease

The prawns transported to AAHL were acclimatised for 4-6 days prior to inoculation. Following inoculation with IMNV, no mortality was observed during the 10-day duration of the experiment. At days 9 and 10 post-inoculation, white, opaque patches were observed in the muscle of IMNV-inoculated prawns (Figure 1). Negative control prawns inoculated with phosphate-buffered saline alone showed no mortality or clinical signs of disease during the 10-day duration of the experiment.

Figure 1a. Typical IMNV muscle lesion at inoculation site of the natural host *L. vannamei*, 5 days post infection.

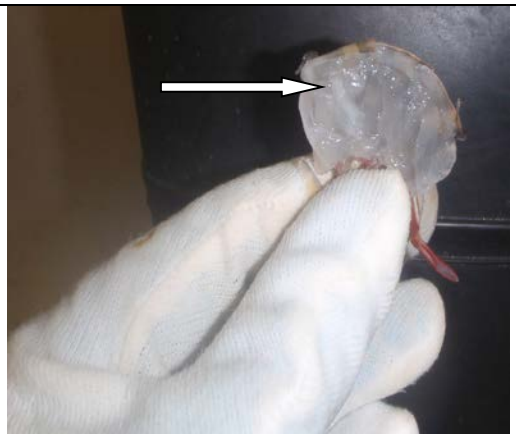

Figure 1b. Photograph of part of the abdominal muscle of IMNV-inoculated (9 dpi) brown tiger prawn dissected to demonstrate an example of the opaque muscle tissue (arrowed), typical of IMNV-infected prawns

2.1.2. PCR Analysis

Both haemolymph and muscle tissue samples were processed for RT-qPCR analysis using the OIE method (OIE, 2014). The C_T values for muscle from individual prawns sampled on each day, is shown in Table 3 and the scatter plots with binomial trend for haemolymph and muscle are shown in Figures 2a and 2b, respectively.

Tissue from all tested negative control (PBS-inoculated) prawns produced a negative result by both real-time RT-qPCR (C_T>45.00) and the OIE-recommended conventional nested RT-PCR (results not shown). One IMNV-inoculated prawn was sampled on 2 dpi and produced a typical amplification curve with a C_T value of 40.20 and is considered positive. All IMNV-inoculated prawns sampled after 2 dpi produced C_T values in the range 12.10-28.45 demonstrating an active infection and increasing levels of virus. The lowest C_T values, in the range 12.00-13.00, only occurred in prawns at 9-10 dpi and it is likely that this range represents the peak viral load for IMNV-infected *F. merguiensis*. IMNV infects tissues of mesodermal origin. The principal target tissues in acute infection are the striated muscles, connective tissues, haemocytes, and the lymphoid organ. While the IMNV C_T values for haemolymph were generally not as low as those found in muscle, the general trend of increasing levels of virus over the later part of the test period was also reflected in the RT-qPCR results for haemolymph.

All samples were tested for the presence of WSSV and IHHNV by qPCR and all produced negative results (C_T>45.00).

Table 3. Analysis of muscle from experimental banana prawns by real-time PCR

Time (days post-infection)	IMNV qPCR C_T values for individual prawns	Uninfected control prawns
0		>45.00
2	40.20	
3	19.85	
3	19.58	
4	14.51	
4	20.66	
5	21.32	
5	21.41	
6	28.34	
6	18.73	
7	15.34	
7	28.45	
8	13.63	
8	16.64	
9	12.75	
9	15.16	>45.00
10	16.03	
10	19.72	
10	18.61	
10	14.48	
10	12.10	
10	12.67	
10	21.35	>45.00

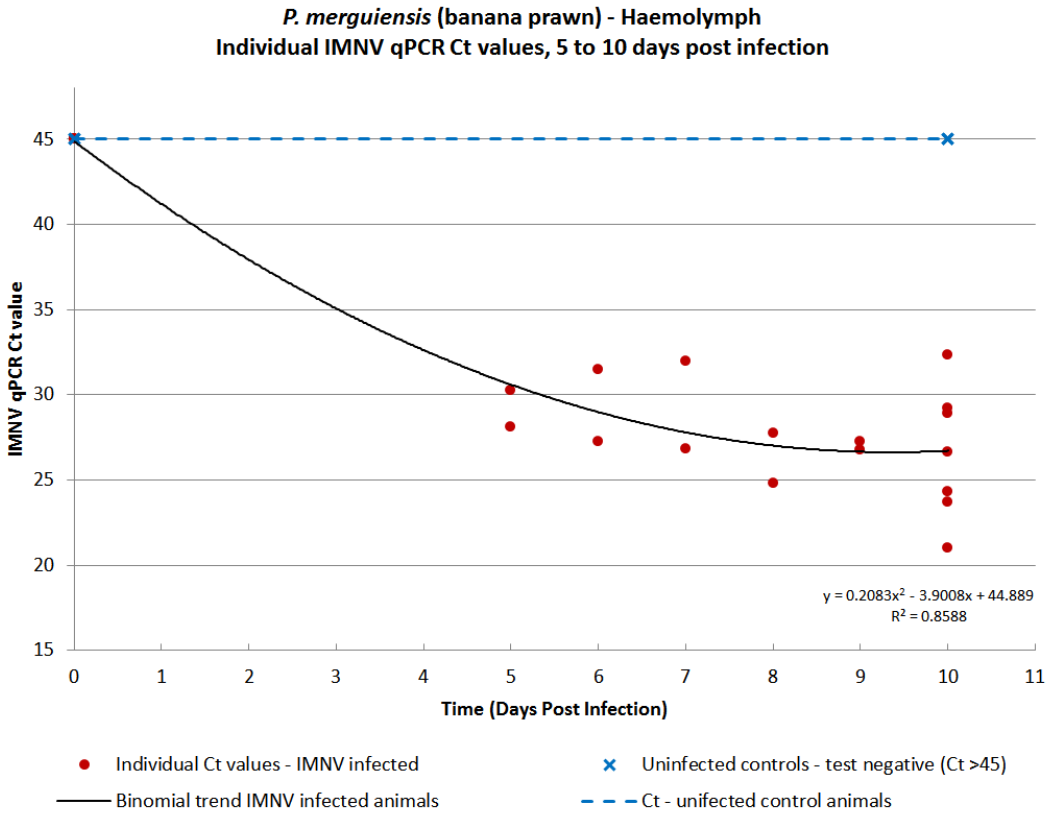

Figure 2a. Graph of real-time PCR results (C_T values) for haemolymph from individual banana prawns sampled on 0-10 dpi.

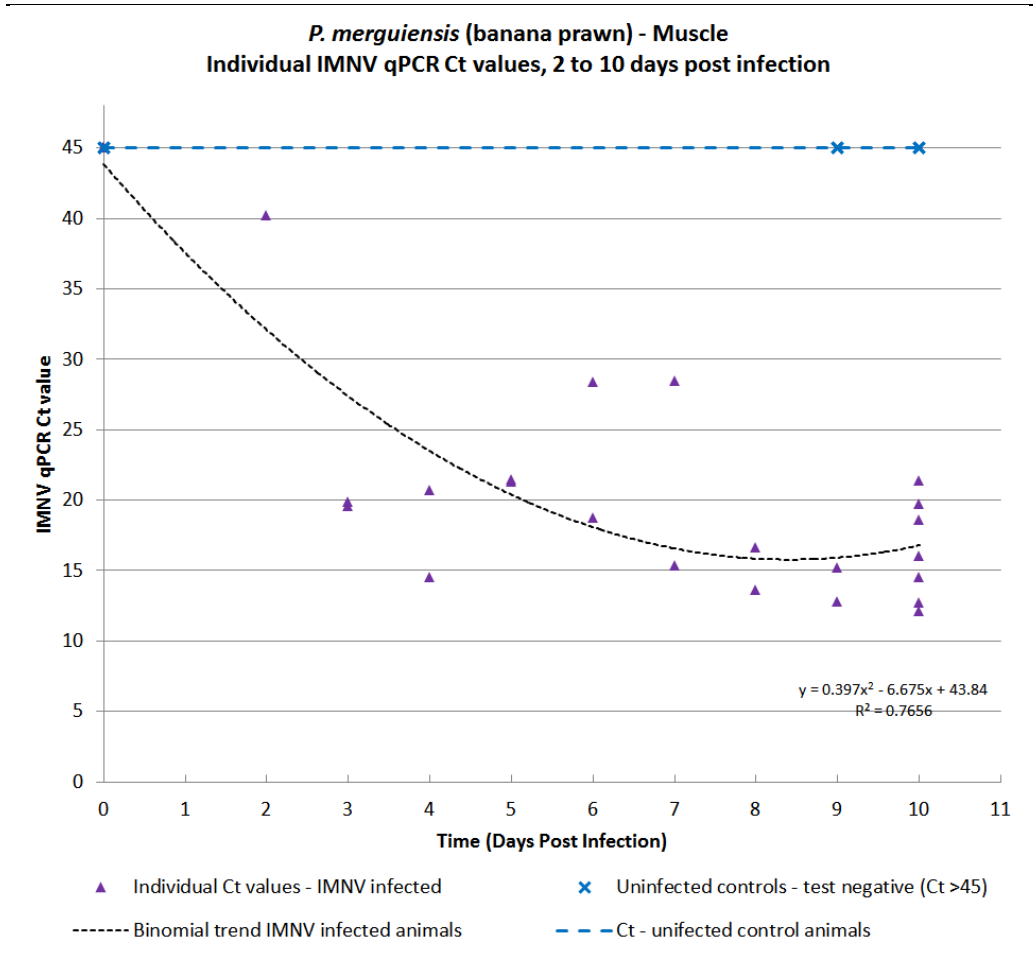

Figure 2b. Graph of real-time PCR results (C_T values) for abdominal muscle from individual banana prawns sampled on 0-10 dpi.

2.1.3. Histopathology and ISH

A sample of four negative control prawns and four IMNV-inoculated prawns were taken for examination for histological lesions and processing for ISH. In the negative control prawns, lesions typical of IMN were not observed in any of the examined sections and ISH produced negative results. Tissue samples from the four IMNV-infected prawns demonstrated coagulation necrosis and infiltration of mononuclear haemocytes, typical of IMN (Table 4 and Figure 3).

Table 4. Summary of histological examination of banana prawn sections

Prawn Id	Time (dpi)	Results of histological examination	ISH results
1 Neg	0	Section of striated muscle with no visible lesions	Negative
2 Neg	0	Section of striated muscle with no visible lesions	Not done
8 Neg	9	Section of striated muscle with no visible lesions	Negative (Figure 3A & B)
9 Neg	9	Section of mostly normal striated muscle. One small area of generalized atrophy of muscle fibres together with occasional degenerate fibres.	Not done
2 IMNV	2	There are at least two affected areas - one small area of acute coagulation necrosis, and another larger area where the lesion is slightly more chronic. The latter appears more cellular, in part due to haemocytic infiltration, and in part due to a higher concentration of muscle cell nuclei (as the muscle fibres degenerate and collapse).	Not done
3 IMNV	3	A couple of foci of coagulation necrosis and degeneration of muscle fibres with increased cellularity due to the presence of haemocytes and some interstitial fibrosis of affected muscle.	one small focus of specific staining
21 IMNV	10	Large area of coagulation necrosis and diffuse infiltration of mononuclear haemocytes. There are also a number of other small discrete foci, sometimes just a few muscle fibres, that are affected. There is heavy infiltration of haemocytes in these regions.	Staining is restricted to areas where lesions are noted histologically. Staining may be diffuse & light, or intense & dark, corresponding to the severity of the lesion (Figure 3C & D).
22 IMNV	10	Far fewer affected foci compared with #21	Not done

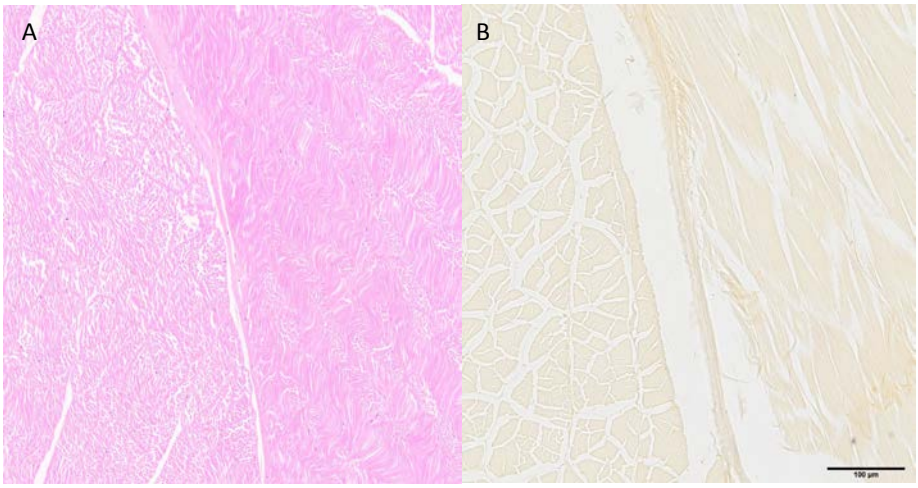

Figure 3a. Photomicrographs of muscle tissue from uninfected control banana prawn sampled at 9 dpi demonstrating normal histology by H&E (A) and negative ISH staining (B).

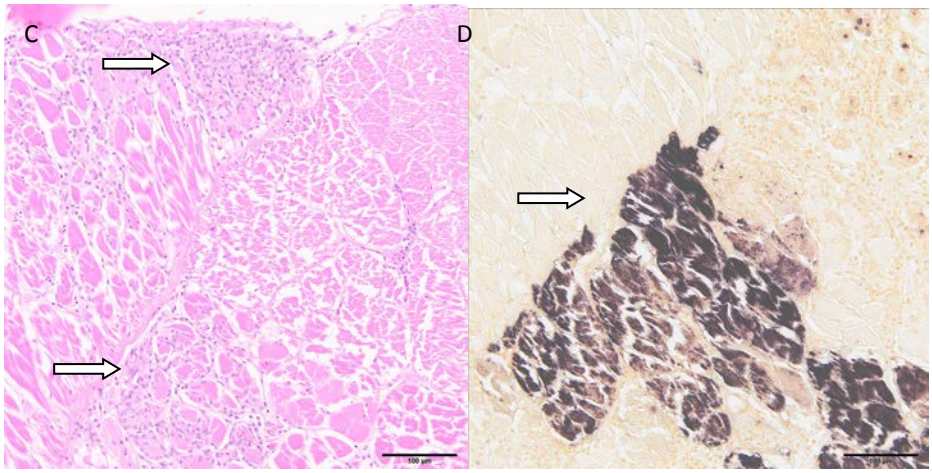

Figure 3b. Photomicrographs of muscle tissue from IMNV-infected banana prawn sampled at 10 dpi demonstrating necrosis and haemocyte infiltration (arrowed) by H&E (C) and positive ISH staining (arrowed) (D).

3. Determination of the susceptibility of brown tiger prawns to IMNV

3.1. Experimental Challenge 1 – Intramuscular injection

3.1.1. Clinical signs of disease

The brown tiger prawns had a shorter trip from Moreton Bay to AAHL and there were less than 6 deaths during the acclimatisation period prior to the infection trial. Following inoculation with IMNV, no mortality was observed during the 10-day duration of the experiment.

3.1.2. PCR Analysis

Again, both haemolymph and muscle tissue samples were processed for RT-qPCR analysis using the OIE method (OIE, 2014). The IMNV C_T values for muscle from individual prawns, is shown in Table 5 and the scatter plots with binomial trend for haemolymph and muscle are shown in Figures 4a and 4b, respectively.

Tissue from all tested negative control (PBS-inoculated) prawns produced a negative result (C_T>45.00). The three prawns sampled on 2 dpi produced typical amplification curves with C_T values in the range of 25.77-36.50 and are considered positive. All prawns sampled after 2 dpi produced C_T values in the range 10.09-29.29 demonstrating that an active infection was on-going. The lowest C_T values, in the range 10.00-15.00, only occurred in prawns at 4-10 dpi and it is likely that this range represents the peak viral load for IMNV-infected *P. esculentus*. As with the banana prawns, the C_T values for IMNV in the brown tiger prawns were higher in the haemolymph samples than observed in the muscle samples with the levels of virus increasing over the test period.

All samples were tested for the presence of WSV and IHHNV by qPCR and all produced negative results (C_T>45.00).

Table 5. Analysis of experimental brown tiger prawns by real-time PCR.

Time (days post-infection)	IMNV qPCR C_T values for individual prawns	Uninfected control prawns
0		>45.00
1		>45.00
1		>45.00
2	26.89	>45.00
2	25.77	
2	36.50	
3	22.84	
3	28.52	
3	29.29	
3	21.48	
4	19.11	
4	25.28	
4	13.19	
4	28.59	
5	15.75	
5	15.27	
5	19.10	
5	28.13	
6	15.76	
6	23.19	
6	20.85	
6	19.20	
7	13.10	
7	17.29	
7	27.36	
7	10.09	
8	16.57	
8	12.54	
8	16.50	
8	19.22	
9	16.00	
9	15.47	
9	20.26	
9	12.68	
10	13.59	
10	19.19	
10	14.92	
10	21.57	
10	28.62	
10	16.34	
10	17.28	
10	12.55	
10	19.10	
10	15.27	
10	18.19	
10	11.35	
10	23.35	
10	28.29	>45.00

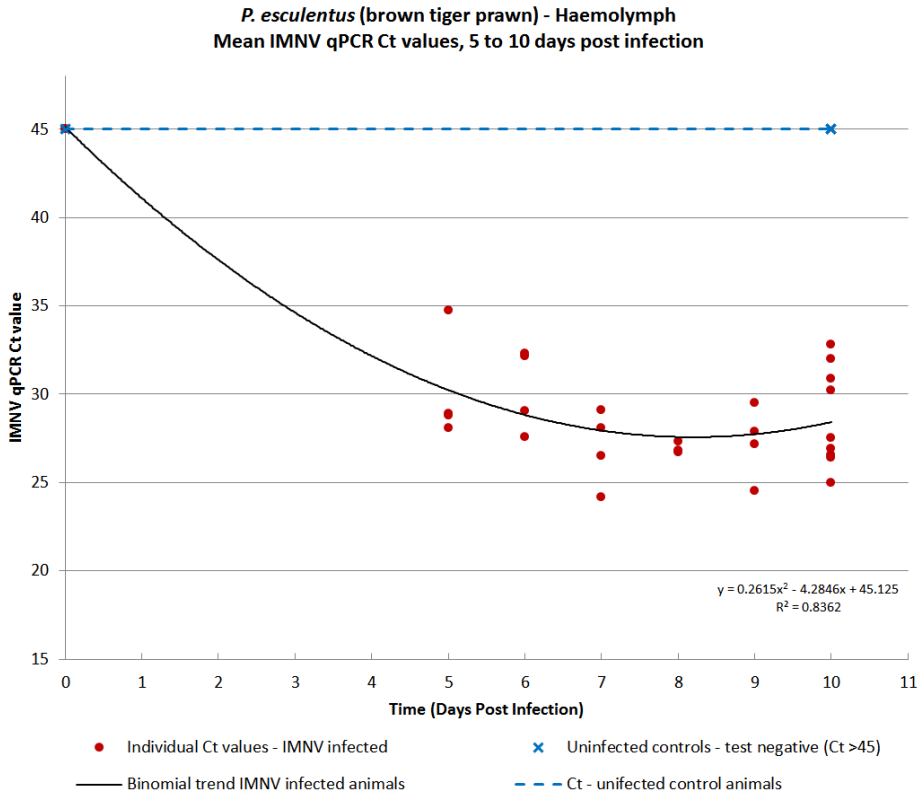

Figure 4a. Graph of real-time PCR results (C_T values) for haemolymph from individual brown tiger prawns sampled on 0-10 dpi.

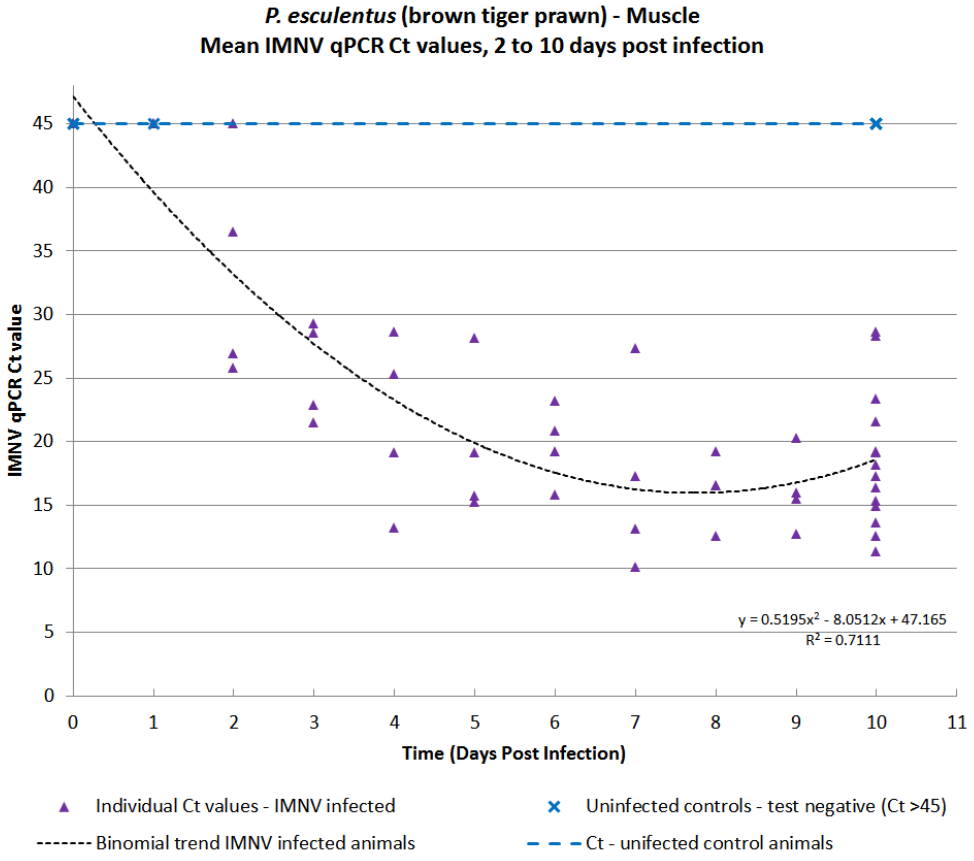

Figure 4b. Graph of real-time PCR results (C_T values) for abdominal muscle from individual brown tiger prawns sampled on 0-10 dpi.

3.1.3. Histopathology and ISH

A sample of four negative control prawns and four IMNV-inoculated prawns were taken for examination for histological lesions and processing for ISH (Table 6). In the negative control prawns, lesions typical of IMN were not observed in any of the examined sections and ISH produced negative results.

Table 6. Summary of histological examination of brown tiger prawn sections

Prawn Id	Time (dpi)	Results of histological examination	ISH results
1 Neg	0	Section of striated muscle with no visible lesions	Negative
2 Neg	0	Section of striated muscle with no visible lesions	Not done
5 Neg	10	Section of striated muscle with no visible lesions	Negative (Figure 5 A & B)
4 IMNV	2	One small focus of mild coagulation necrosis of the muscle.	No clearly defined staining.
7 IMNV	3	A couple of tiny foci of coagulation necrosis.	Not done
38 IMNV	10	Multiple foci of peracute to chronic coagulation necrosis. The chronic foci are not quite as severe as in the banana prawns at 10 dpi.	The peracute foci are best recognized by ISH.
46 IMNV	10	More extensive areas of acute coagulation necrosis, and occasional small foci of a more chronic lesion.	ISH staining seems to be mainly restricted to the very acutely affected regions (Figures 5 C & D).

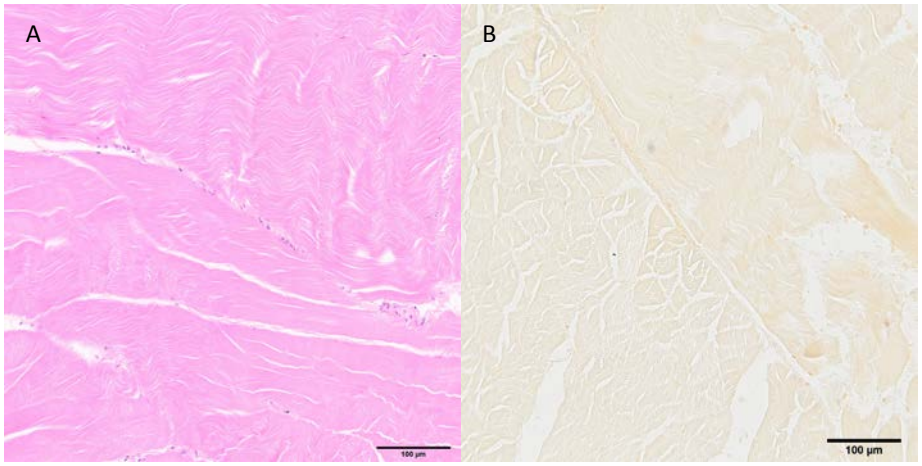

Figure 5a. Photomicrographs of muscle tissue from uninfected control brown tiger prawn sampled at 10 dpi showing normal histology by H&E (A) and negative ISH staining (B).

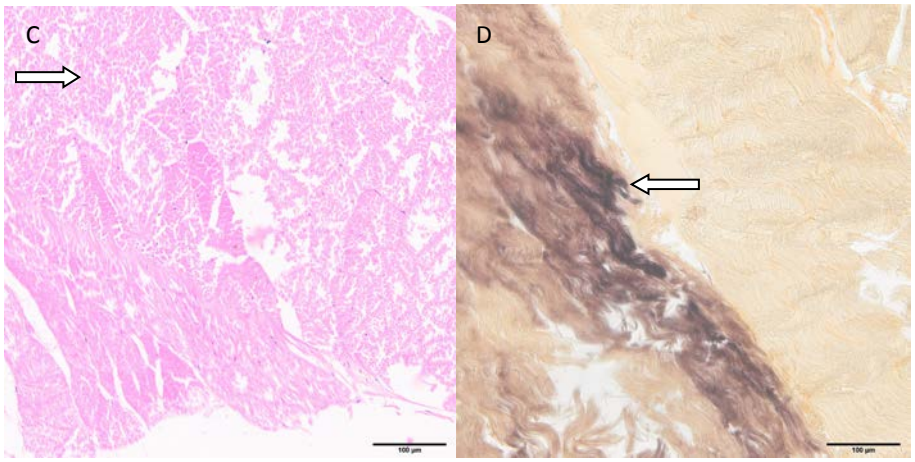

Figure 5b. Photomicrographs of muscle tissue from IMNV-infected brown tiger prawn at 10 dpi, extensive necrosis (arrowed) by H&E (C) and positive ISH staining (arrowed) (D).

4. Determination of the susceptibility of Pacific white prawns to IMNV

Using the same experimental protocol, the same inoculum (stored at MCBAD) was used to infect the highly susceptible host, Pacific white prawn (*L. vannamei*) at MCBAD. The prawns were monitored on a daily basis and samples taken when IMNV-inoculated prawns showed disease signs (e.g. opaque patches in abdominal muscle), at 5-8 dpi (Figure 6). The series of photographs taken on 5-8 dpi demonstrate the development of the opaque patches on the abdominal muscle in the IMNV-inoculated prawns. None of the negative control prawns demonstrated this disease sign.

Real-time PCR facilities were not available at MCBAD and so the OIE conventional nested RT-PCR test was used to confirm presence of IMNV (Figure 7). Results confirmed that the inoculum used for all experimental infections at AAHL and at MCBAD was infectious and pathogenic for the Pacific white prawn (*L. vannamei*). It is interesting to note that the sample from an IMNV-inoculated prawn produced relatively intense amplicons in both the primary (328 bp) and the nested steps (139 bp) of the RT-PCR, indicating a high level of infection. With the positive control sample, a clear amplicon was produced in the nested step with the primary step amplicon relatively faint compared with the experimentally inoculated prawn sample. No amplicons were produced in the no template control.

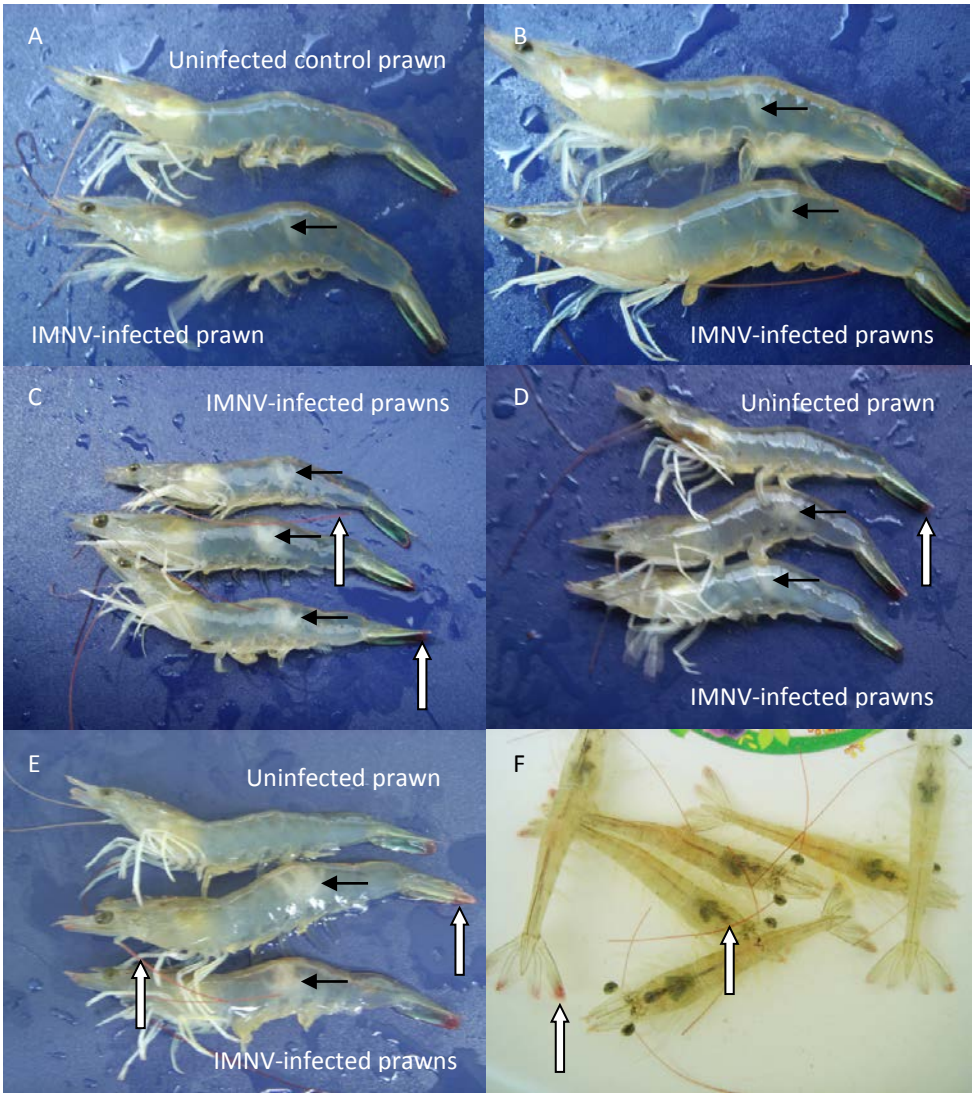

Figure 6. Photographs of experimental Pacific white prawns demonstrating, in IMNV-infected prawns, opaque patches (black arrows) in the abdominal muscle, and reddening (white arrows), particularly in the tail and antennae, at 5 (A and B), 6 (C), 7 (D) and 8 (E and F) dpi.

Lane M: 100 bp DNA ladder
Lane 1: Positive control 1st step RT-PCR
Lane2: Positive control 2nd step RT-PCR
Lane 3: Negative control 1st step RT-PCR
Lane 4: Negative control 2nd step RT-PCR
Lane 5: IMNV-inoculated 1st step RT-PCR
Lane 6: IMNV-inoculated 2nd step RT-PCR

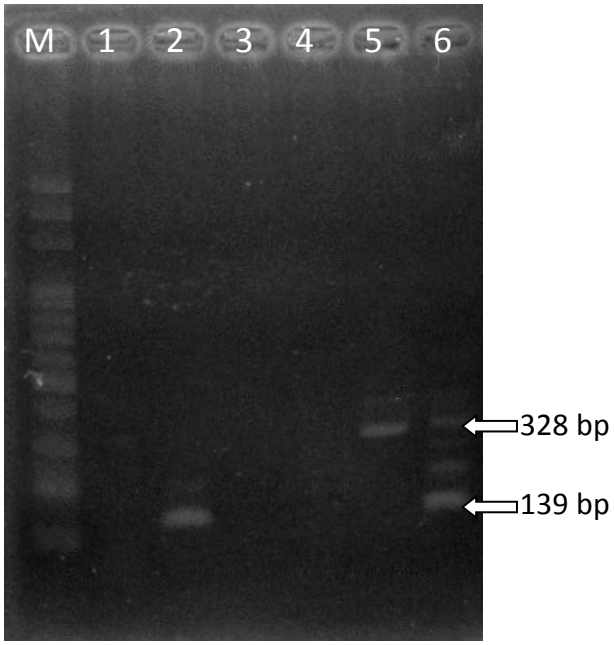

Figure 7. Pacific white prawns: Photograph of IMNV conventional RT-PCR results.

Tissues and haemolymph from these experimentally infected prawns provide a useful source of positive control and reference material for future experiments and diagnostic testing.

5. Trial 2 – Transmission by Water-Borne Exposure and Feeding of Infected Tissue

With respect to the guidelines provided by the OIE for determining the susceptibility of host species, all screening and identification of samples for IMNV was conducted in accordance with methods described in the disease chapters in the Chapter 2.2.3 of the Aquatic Manual. Recognising the limitations imposed in using limited animal numbers held under laboratory conditions, the experiments in the second trial included 3 challenge methods which were intended to simulate natural routes of infection including water borne exposure and exposure by feeding. The RT-qPCR, histology and *in situ* hybridisation undertaken provided a combination of criteria to assess infection in the test species and provided evidence of transmission of the viable organism, evidence of multiplication and confirmed pathological changes associated with the infection in tissues known to be targeted by IMNV.

5.1. Molecular Analysis

5.1.1. IMNV in Prawns used for Feeding and Co-habitation

IMNV was confirmed by RT-qPCR in all banana prawns used as a source of tissue for feeding (Table 7). Similarly, replication of IMNV was confirmed in both banana and brown tiger prawns inoculated with IMNV for the purpose of co-habitation (Tables 8a/b). The proximal segment of an anterior pleopod was used as a source of nucleic acid for this testing. As these animals were inoculated i.m. in the 2nd abdominal segment, the IMNV detected in the pleopod was unlikely to represent the detection of the inoculum. The C_T values of these samples ranged from 18.96 to 27.45 with a mean of 23.43. These elevated levels confirmed the IMNV was viable and replicating in these animals. Similarly, the animals used for short-term co-habitation (Table 8a/b) showed elevated IMNV levels, indicating that the virus was viable and replicating.

Table 7. IMNV RT-qPCR confirmation of inoculated *F. merguiensis* used for feeding (8 days post IM inoculation)

SAN	Sample	Days PI	CT (mean)	CT SD	Quantity (mean)*
14-03237	F-01	8	21.63	0.08	1453179.88
14-03237	F-02	8	24.90	0.06	86250.05
14-03237	F-03	8	23.45	0.07	301282.00
14-03237	F-04	8	25.19	0.09	67021.50
14-03237	F-05	8	21.39	0.07	1782133.00
14-03237	F-06	8	24.29	0.02	145257.13
14-03237	F-07	8	21.31	0.35	1967963.50
14-03237	F-08	8	26.20	0.07	27857.16
14-03237	F-09	8	27.45	0.16	9534.72
14-03237	F-10	8	24.46	0.15	125877.25
14-03237	F-11	8	20.31	0.01	4567015.00
14-03237	F-12	8	18.96	0.05	14660818.00
14-03237	F-13	8	23.94	0.04	197134.69
14-03237	F-14	8	23.95	0.08	194852.00
14-03237	F-15	8	21.10	0.19	2311564.75
14-03237	F-16	8	26.32	0.03	25082.97
14-03237	F-17	8	26.84	0.11	16125.63
14-03237	F-18	8	23.78	0.01	226585.03
14-03237	F-19	8	24.04	0.02	181499.83
14-03237	F-20	8	22.41	0.05	741433.25
14-03237	F-21	8	19.45	0.01	9584104.00
14-03237	F-22	8	24.17	0.03	161929.50

*Quantity (mean) = the mean copy number per reaction (n=2) calculated from a standard curve generated by serial dilution of a plasmid constructed according to the IMNV Chapter in the *Aquatic Manual* (OIE, 2014).

Table 8a. RT-qPCR confirmation of IMNV-inoculated *F. merguiensis* used for co-habitation

SAN	Sample	Days PI	CT (mean)	CT SD	Quantity (mean)
14-03237	_001	14	18.10	0.05	8192329.50
14-03237	_002	14	17.63	0.07	11259066.00
14-03237	_003	18	17.06	0.16	16536831.00
14-03237	_004	20	29.50	0.09	3815.99
14-03237	_005	20	27.41	0.19	15596.11
14-03237	_006	20	30.85	0.31	1558.40
14-03237	_007	20	20.16	0.23	2011780.75
14-03237	_008	20	26.29	0.02	42756.95
14-03237	_009	20	Und		
14-03237	_010	20	28.12	0.05	13543.59
14-03237	_011	20	28.40	0.54	11685.09
14-03237	_012	20	22.90	0.09	358596.56
14-03237	_013	20	Und		

Table 8b. RT-qPCR confirmation of IMNV-inoculated *P. esculentus* used for co-habitation

SAN	Sample	Days PI	CT (mean)	CT SD	Quantity (mean)
14-03242	_001	2	Und		
14-03242	_002	2	20.11	-	2065594.63
14-03242	_003	9	28.04	0.23	14308.17
14-03237	_014	9	32.78	0.04	726.85
14-03237	_015	9	22.83	0.36	378591.78
14-03242	_006	11	20.43	0.67	1765799.75
14-03242	_007	15	16.84	0.24	16101268.00

5.1.2. Exposure by Co-habitation with IMNV-Infected Animals (transmission by water/fomites)

Over the course of the co-habitation experiment, samples of both test species were taken every second day (Table 10). Although previous experiments had shown that IMNV would replicate in both species following parenteral inoculation the rate of shedding of virus into the water from infected animals was unknown, the survival of virus in water was also unknown. In order to confirm IMNV was released into the water from infected animals, samples of water were collected over the course of the experiment. The IMNV RT-qPCR analysis of water samples is summarised in Table 9.

Although IMNV was detectable in the water of one banana prawn tank (Tank 7) at a low level in the water at 12 days (C_T 36.39), this was an isolated observation and virus was only reliably detected at 20 days. Taking into account the sample volumes and dilutions used for testing, the estimated number of viral copies in tank 7 (banana prawns) at 20 days was 94,533 copies per ml and in tank 9 (brown tiger prawns) was 2,982 copies per ml. Recognising that these estimates do not provide evidence of the levels of *infectious* virus, the results of water screening indicated that significant levels of virus were probably not present in the water until sometime after day 16. The relative short exposure time provides some explanation for the observation that only 3 of 22 banana prawns were found positive and one of 26 brown tiger prawns was positive by RT-qPCR after 20 days exposure. Although RT-qPCR showed that the levels of virus in these animals was very low, one of the banana prawns (sample 041) was also positive by the OIE conventional nested RT-PCR (Figure 8 Lane 3) and IMNV sequence was confirmed from the 1st step amplification product. Histological lesions were also observed in muscle tissues, but virus was not detectable by *in situ* hybridisation (14-03238-20). The combination of positive PCR and presence of muscle lesions in histology confirmed that this banana prawn was susceptible to infection by this procedure. The single RT-qPCR positive brown tiger prawn (14-03243-21) was infected at a very low level, no conventional PCR product was obtained for sequencing and although histological lesions were observed, *in situ* hydridisation was negative for this animal. The overall results for the co-habitation trial with brown tiger prawns are consistent with the lower levels of virus in the water compared to the banana prawns.

Table 9. IMNV RT-qPCR analysis of water from co-habitation Tank 7 (*F. merguiensis*) and Tank 9 (*P. esculentus*) at 12, 16 and 20 days

Tank 7 - *F. merguiensis* (banana prawn)

SAN	Sample	Days PI	CT (mean)	CT SD	Quantity (mean)
14-03238	water d12	12	36.39		75.40
14-03238	water d16	16			
14-03238	water d20	20	24.39	0.44	143690.52

Tank 9 - *P. esculentus* (brown tiger prawn)

14-03243	water d12	12	Und		
14-03243	water d16	16	Und		
14-03243	water d20	20	29.87	0.10	4534.10

Table 10. IMNV RT-qPCR test results from sequential samples of test species co-habited with IMNV-infected animals

F. merguiensis (banana prawn) - co-habitation						*P. esculentus* (brown tiger prawn) - co-habitation				
SAN	Sample	Days PI	CT (mean)	Quantity (mean)		SAN	Sample	Days PI	CT (mean)	Quantity (mean)
14-03238	_001	2	Und			14-03243	_001	2	Und	
14-03238	_002	2	Und			14-03243	_002	2	Und	
14-03238	_006	6	Und			14-03243	_003	2	Und	
14-03238	_007	8	Und			14-03243	_004	2	Und	
14-03238	_008	8	Und			14-03243	_005	2	Und	
14-03238	_009	10	Und			14-03243	_006	6	Und	
14-03238	_010	10	Und			14-03243	_007	6	Und	
14-03238	_011	12	Und			14-03243	_008	8	Und	
14-03238	_012	12	Und			14-03237	_009	8	Und	
14-03238	_013	14	44.49	0.06		14-03243	_010	8	Und	
14-03238	_014	14	Und			14-03243	_011	10	Und	
14-03238	_015	16	Und			14-03243	_012	12	Und	
14-03238	_016	16	Und			14-03243	_013	12	Und	
14-03238	_017	18	Und			14-03243	_014	14	Und	
14-03238	_018	18	Und			14-03243	_015	14	Und	
14-03238	_019	20	41.22	0.49		14-03243	_016	16	Und	
14-03238	_020	20	33.93	160.31		14-03243	_017	16	Und	
14-03238	_021	20	Und			14-03243	_018	18	Und	
14-03238	_022	20	Und			14-03243	_019	18	Und	
						14-03243	_020	20	Und	
						14-03243	_021	20	44.28	0.67
						14-03243	_022	20	Und	
						14-03243	_023	20	Und	
						14-03243	_024	20	Und	
						14-03243	_025	20	Und	
						14-03243	_026	20	Und	

5.1.3. Exposure by Feeding of Infected Tissues (Simulated Predation)

Exposure by feeding produced more RT-qPCR-positive banana prawns than brown tiger prawns where 10 of 20 (50%) banana prawns were test positive by RT-qPCR, compared with only 4 brown tiger prawns which were found positive during the course of testing. As previously mentioned, the brown tiger prawns were significantly more stressed than the banana prawns in this series of experiments and only a few animals were observed to actively feed at the time when infected tissues were presented. As these animals did not ingest virus infected tissue to the same extent as the actively feeding banana prawns it was clear that these inappetent brown tiger prawns did not provide an ideal assessment of the potential susceptibility of this species by feeding.

Subsequent testing of selected IMNV RT-qPCR-positive banana prawns further confirmed the presence of IMNV by OIE conventional PCR and DNA sequencing (Table 13, Figure 8), and characteristic histological lesions and positive IMNV *in situ* hydridisation (Figure 11) was observed in animal 055 (14-03239-12).

Several brown tiger prawns were also examined, and conventional PCR and sequencing confirmed IMNV in these animals (Table 13) with histological lesions in the abdominal muscle also observed. The low levels of virus in these animals precluded confirmation by *in situ* hydridisation. Nevertheless the combination of positive molecular results with characteristic histological lesions in the target tissue provided good evidence of the susceptibility of both species by feeding of infected tissues and therefore susceptibility by predation in a natural setting.

Table 11. IMNV RT-qPCR test results of sequential samples from animals fed IMNV-infected tissues

F. merguiensis **(banana prawn) - feeding**						***P. esculentus*** **(brown tiger prawn) - feeding**				
SAN	**Sample**	**Days PI**	**Cт (mean)**	**Quantity (mean)**		**SAN**	**Sample**	**Days PI**	**Cт (mean)**	**Quantity (mean)**
14-03239	_001	2	36.66	16.67		14-03244	_001	2	Und	
14-03239	_002	2	Und			14-03244	_002	3	31.26	2252.67
14-03239	_003	3	37.80	7.41		14-03244	_003	4	44.76	0.49
14-03239	_004	4	31.36	721.37		14-03244	_004	4	36.95	70.81
14-03239	_005	4	32.22	395.94		14-03244	_005	5	Und	
14-03239	_006	5	44.53	0.06		14-03244	_006	5	Und	
14-03239	_007	5	30.31	1516.35		14-03244	_007	6	Und	
14-03239	_008	6	Und			14-03244	_008	6	Und	
14-03239	_009	6	Und			14-03244	_009	7	40.37	4.54
14-03239	_010	7	29.86	2087.06		14-03244	_010	7	Und	
14-03239	_011	8	Und			14-03244	_011	8	Und	
14-03239	_012	8	29.71	2355.89		14-03244	_012	8	Und	
14-03239	_013	9	Und			14-03244	_013	9	Und	
14-03239	_014	9	37.26	24.32		14-03244	_014	9	Und	
14-03239	_015	10	Und			14-03244	_015	10	Und	
14-03239	_016	10	Und			14-03244	_015	10	Und	
14-03239	_017	11	Und			14-03244	_016	11	Und	
14-03239	_018	11	Und			14-03244	_016	11	Und	
14-03239	_019	12	32.50	374.89		14-03244	_017	11	Und	
14-03239	_020	12	Und			14-03244	_018	12	Und	
						14-03244	_019	12	Und	
						14-03244	_020	13	Und	

5.1.4. Bath Exposure

The bath exposure was intended to provide additional support for susceptibility by water-borne transmission should the release of virus into the water prove unreliable in the co-habitation experiment. In this case the virus in the water was assured at the beginning of the 20 day test period.

Only 5 individuals of each species were used. As previously mentioned, the brown tiger prawns were stressed and 2 animals died within days of the initial bath. Of the remaining animals 3 of 5 banana prawns were RT-qPCR positive and IMNV was further confirmed by conventional PCR (Figure 8, Lane 11) and sequencing. The individual prawns of each test species assessed with the highest levels of IMNV by real-time PCR were also positive by conventional IMNV RT-PCR, and had characteristic histological lesions and showed positive staining with the IMNV probe in *in situ* hybridisation. While there might be some question as to the circumstances that might lead to high levels of virus in the water under natural conditions, both test species (*F. merguiensis* and *P. esculentus*) were susceptible to IMNV infection following this bath exposure.

Table 12. IMNV RT-qPCR results of samples from test animals following IMNV bath

F. merguiensis (banana prawn) - bath						*P. esculentus* (brown tiger prawn) - bath				
SAN	Sample	Days PI	CT (mean)	Quantity (mean)		SAN	Sample	Days PI	CT (mean)	Quantity (mean)
14-03250	_001	1	39.10	5.99		14-03252	_003	11	34.32	85.48
14-03250	_002	19	Und			14-03252	_004	20	35.78	39.30
14-03250	_003	20	Und			14-03252	_005	20	25.52	6005.10
14-03250	_004	20	44.11	0.20						
14-03250	_005	20	20.80	1403919.00						

Table 13. Summary of selected samples and testing of individuals from all exposure methods in Trial 2

Treatment	Species	Sample No.	qPCR Mean C_t	Lane No.	RT-PCR 1st step	RT-PCR 2nd step	Seq	SAN Hist/ISH	Histology (Lesions)	ISH Staining	Comment
Co-habitation	Banana	**_034**	44.49	**1**	neg	neg	NA				
Co-habitation	Banana	**_040**	41.22	**2**	neg	neg	NA				
Co-habitation	Banana	**_041**	33.93	**3**	pos	pos	1st	**14-03238-20**	Yes	neg	
Feeding	Banana	**_053**	29.82	**4**	pos	pos	1st	**14-03239-10**	No	neg	
Feeding	Banana	**_055**	29.51	**5**	pos	pos	1st	**14-03239-12**	Yes	pos	
Co-habitation	Brown Tiger	**_084**	44.28	**6**	neg	neg	NA	**14-03243-21**	Yes	neg	
Feeding	Brown Tiger	**_091**	30.88	**7a**	pos	pos	1st				Mort. No histo/ISH
Feeding	Brown Tiger	**_098**	38.61	**7b**	nd	nd	NA	**14-03244-09**	Yes	neg	replacement
Feeding	Brown Tiger	**_092**	44.56	**8**	pos	pos	1st	**14-03244-03**	No	neg	
Feeding	Brown Tiger	**_093**	36.19	**9**	neg	neg	NA				Mort. No histo/ISH.
Bath	Banana	**_114**	39.10	**10**	neg	neg	NA				
Bath	Banana	**_118**	20.72	**11**	pos	pos	1st	**14-03250-05**	Yes	pos	
Bath	Brown Tiger	**_119**	33.73	**12**	neg	neg	NA				
Bath	Brown Tiger	**_121**	25.41	**13**	neg	pos	2nd	**14-03252-05**	Yes	pos	
		_147	24.08	**14**	pos	pos	1st				water tank7 d20
		_150	29.79	**15**	pos	pos	1st				water tank9 d20
Controls											
	Banana		12.09		pos	pos		**13-02769-21**	Yes	pos	positive control
	Brown Tiger		neg		neg	neg		**13-02770-01**	No	neg	negative control
	Banana		11.35		pos	pos		**13-02771-46**	Yes	Pos	positive control

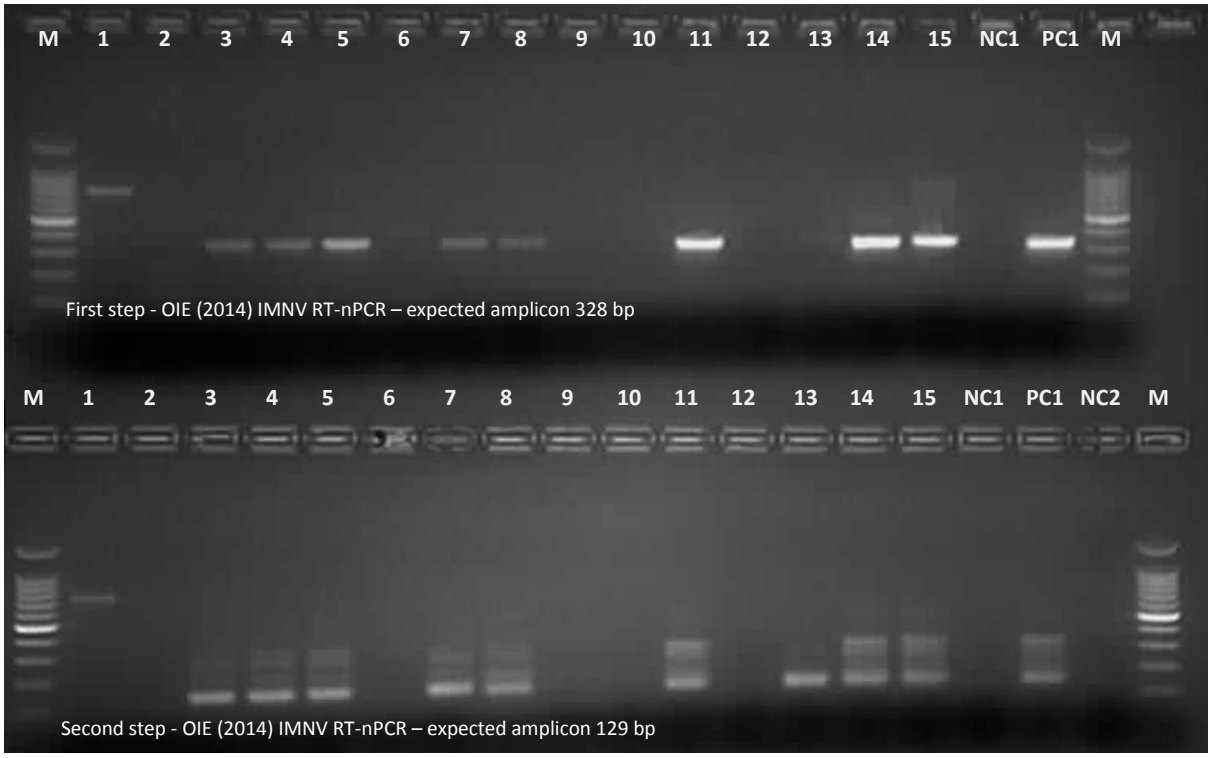

Figure 8. OIE IMNV conventional nested RT-PCR of select high C_T samples representative of each treatment group for sequence confirmation of PCR products. Lane numbers and samples are shown in Table 12 (above)
PC = positive control, NC = negative control, M = 100 bp markers (Promega)

5.2. IMNV Sequence Confirmation

Nine samples (summarised in Table 12 and shown in Figure 8) were selected for DNA sequencing. The samples were representative of both test species and the 3 treatment groups with the exception of the co-habited brown tiger prawns where only one positive sample with a very high C_T was identified by real-time PCR. No conventional PCR product was obtained from this sample. The virus-associated PCR amplicons from the water samples taken from tanks 7 and 9 were also included. IMNV was confirmed by BLAST analysis of sequence from all 9 samples tested.

5.3. Testing of Negative Control Animals

All samples from unexposed negative control animals screened for IMNV by OIE RT-qPCR, OIE conventional RT-PCR, histology or IMNV *in situ* hybridisation were test negative.

5.4. Histopathology and ISH

5.4.1. Banana prawn – Bath treatment 14-03250-05-03 (sampled at 20 d post-exposure)

The H&E section reveals multiple foci of chronic myositis and in one of these foci there is some acute coagulation necrosis and oedema of muscle bundles on the periphery of the chronic lesion. The chronic areas are more cellular due to haemocytic infiltration and fibrosis, in addition to a higher concentration of muscle cell nuclei (as the muscle fibres degenerate and collapse). These lesions would be consistent with the prawn being infected with IMNV very early in the course of the 20 day trial, but with more acute lesions continuing to develop throughout the trial as the virus continued to spread throughout the muscle. There appears to be some sort of degenerating parasite in the muscle section.

The corresponding ISH section is characterized by very little staining of the chronically-affected muscle; the most intense staining occurs in acutely-affected oedematous areas of muscle and in the early stages of coagulation necrosis (Figure 8).

5.4.2. Brown tiger prawn – Bath treatment 14-03252-05-03 (sampled at 20 d post-exposure)

In the H&E section, while there are foci of mild chronic myositis scattered throughout the section, there are more regions of more acute coagulation necrosis. Only the latter stain intensely by ISH. These findings suggest that, despite being exposed to infectious virus for the same time as 14-03250, this prawn did not actually suffer widespread infection until later (Figure 9).

5.4.3. Banana prawn – Feeding Group 14-03239-12-03 (sampled at 8 d post-exposure)

In the H&E section, there are few readily recognizable muscle lesions. However, there is a region of the ISH section where there is moderate staining, and re-examination of the equivalent region in the H&E section suggests that there is early coagulation necrosis of some fibers (Figure 10).

As in 14-03250, there appears to be a degenerating parasite in the section (possibly in the body cavity?).

5.4.4. Summary - Histology and in situ hybridisation

Overall, it appears that, following exposure by a natural route of infection, the first acute lesions were only beginning to develop at 8 d post-exposure (in one prawn examined at this time-point). These lesions could be detected with difficulty on H&E sections, and were only just detected by ISH. In one of the two prawns that was sacrificed at 20 d post-exposure, early lesions seemed to have progressed, and muscle was characterized largely by multifocal chronic lesions. Acute foci were present less commonly, often at the periphery of the chronic lesions. ISH barely stained the chronic lesions, but was much stronger in acute lesions. For the remaining prawn sacrificed at 20 d post-exposure, widespread infection seems to have occurred later because most lesions are of a more acute nature.

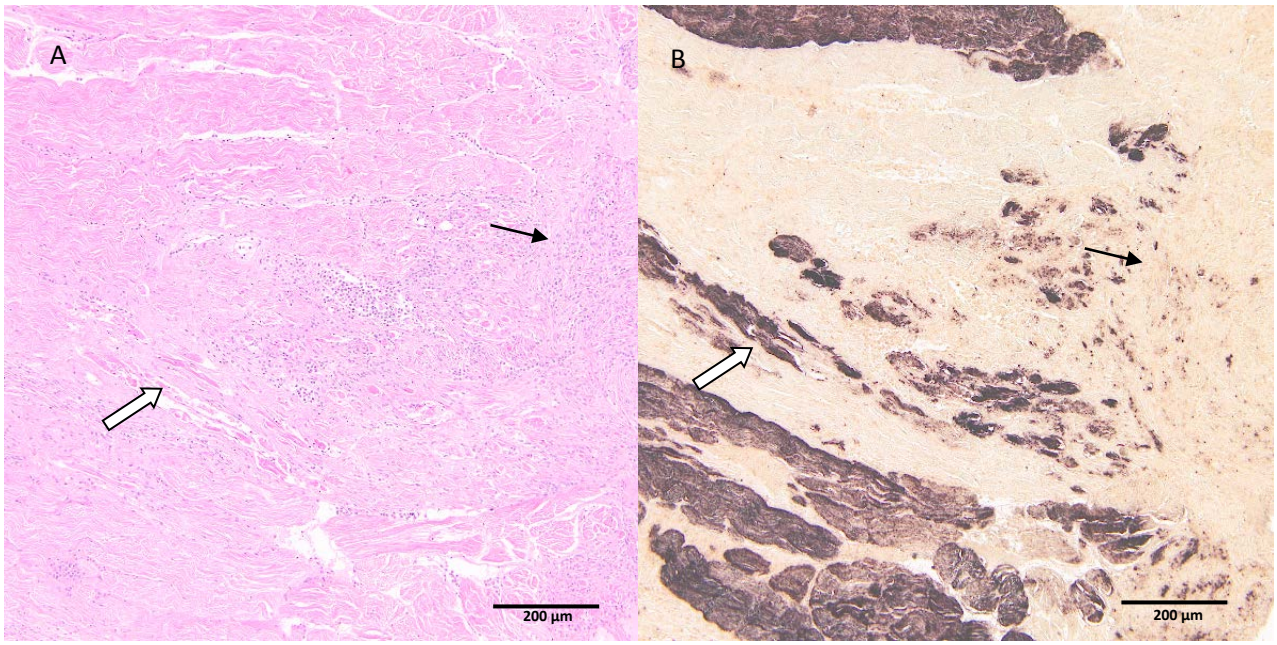

Figure 9. Photomicrograph (x10) of serial sections of muscle tissue from a banana prawn (14-3250-05) exposed to IMNV for 20 days demonstrating acute necrosis (block arrow) and chronic lesions (thin arrow) by H&E (A) and positive ISH staining (B).

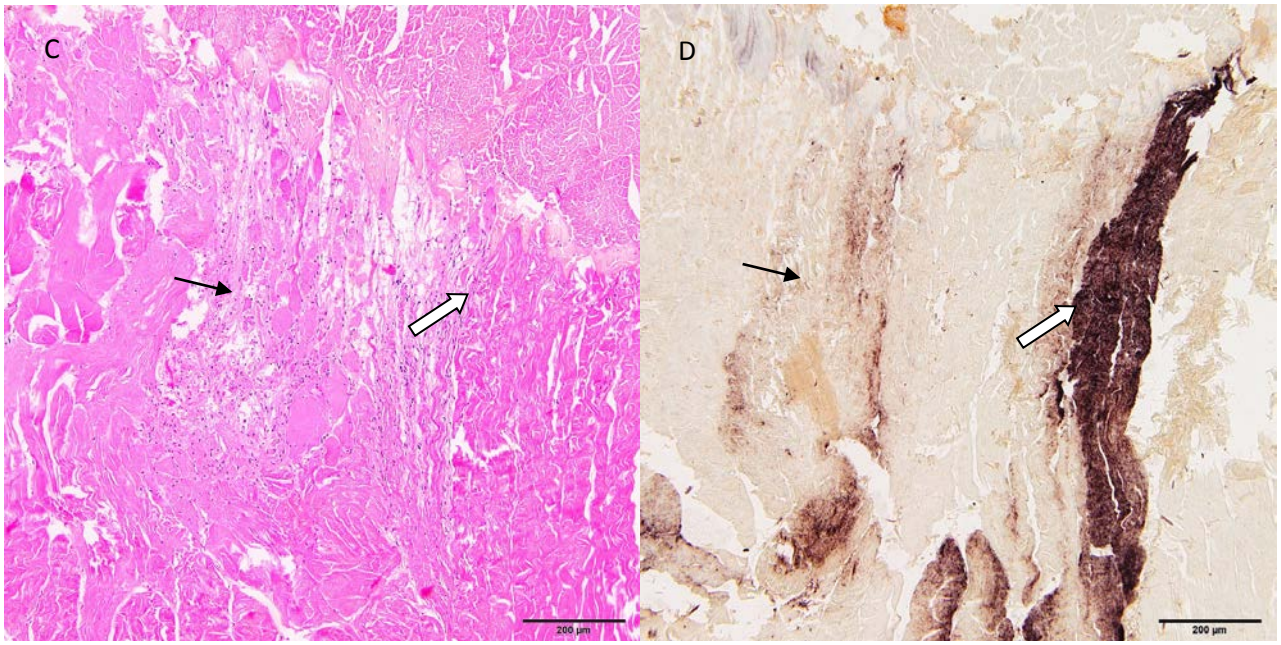

Figure 10. Photomicrograph (x10) of sections of muscle tissue from a brown tiger prawn (14-3252-05) exposed to IMNV for 20 days demonstrating acute necrosis (block arrow) and chronic lesions (thin arrow) by H&E (C) and positive staining by ISH (D).

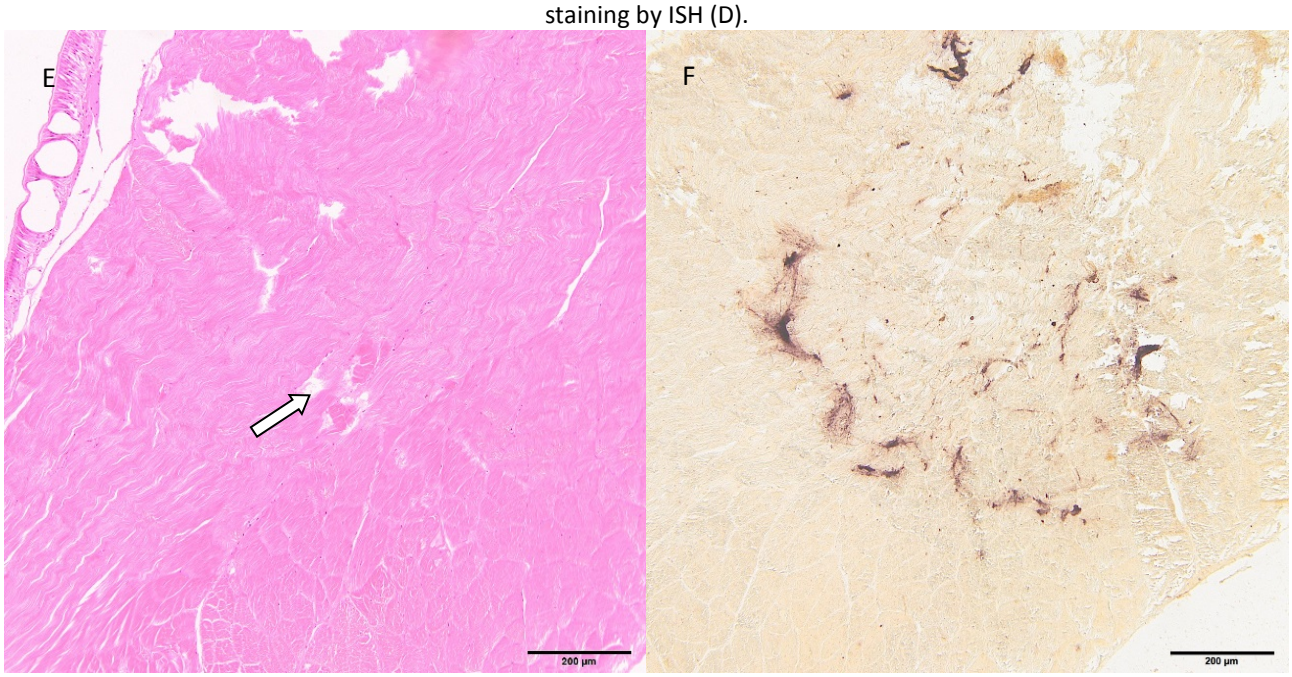

Figure 11. Photomicrograph (x10) of muscle tissue from a banana prawn (14-3239-12) exposed to IMNV by feeding, 12 days post-exposure (E) with a small area of acute necrosis (block arrow) in H&E and positive staining by ISH (D).

Conclusion

In order to achieve the primary objective of this project, viz. determination of the susceptibility of banana prawns (*Fenneropenaeus merguiensis*) and brown tiger prawns (*Penaeus esculentus*) to IMNV, it was necessary to undertake a substantial amount of work with the Pacific white prawn (*Litopenaeus vannamei*) which is the natural host and is not available in Australia. Thus part of the project was undertaken in Indonesia where the host species is available and the disease has been reported. The Fish Health and Environment Laboratory, Main Centre for Brackishwater Aquaculture Development (CBAD), Jepara, Central Java, Indonesia has the facilities and capability to assist with this project and MCBAD staff were keen to collaborate on this project.

Thus through this collaboration it was possible to generate and characterise an infectious IMNV inoculum that was free from other viral pathogens of prawns and to import it into the high-level bio-secure facility at the CSIRO Australian Animal Health Laboratory at Geelong. This IMNV inoculum was used for experimental infections at AAHL of farmed banana prawns and wild-caught brown tiger prawns, and at MCBAD of positive control Pacific white prawns.

Results demonstrated that when the inoculum was inoculated (i.m.) into the experimental prawns it was infectious, and caused disease, in all three species of prawns.

A second series of experiments was used to demonstrate the susceptibility of banana and brown tiger prawns to IMNV infection via simulated natural routes of transmission. All procedures for pathogen identification and confirmation were carried out in accordance with guidelines set out in Chapter 2.2.3 of the *Aquatic Manual* (OIE, 2014). Three non-invasive procedures were used to emulate natural routes of infection, including water-borne transmission and predation. A combination of molecular testing (PCR/sequencing and ISH) and histology was used to confirm infection and demonstrate virus replication in both banana prawns (*Fenneropenaeus merguiensis*) and brown tiger prawns (*Penaeus esculentus*).

Implications

This investigation has demonstrated that the two commercial species of prawns of Australian origin, *Fenneropenaeus merguiensis* and *Penaeus esculentus*, are susceptible to infection with the exotic virus IMNV. This extension of the known host range for this virus is important information for industry and regulators to be aware of when considering importation of commodity prawns from countries where IMNV is considered endemic.

Recommendations

Prior to this study, it was known that IMNV causes significant disease outbreaks and mortalities in farmed populations is *Penaeus vannamei* (commonly called the Pacific white shrimp or white leg shrimp) (Lightner *et al.,* 2004; Nunes *et al.,* 2004) and that the Pacific blue shrimp, *P. stylirostris*, and the black tiger shrimp, *P. monodon* can been infected experimentally with IMNV (Tang *et al.,* 2005). Following this study we now know that the banana prawn (*Fenneropenaeus merguiensis*) and the brown tiger prawn (*P. esculentus*) can also be infected experimentally with IMNV (via natural routes of transmission). The results of this study should be published in a peer-reviewed scientific journal to inform the international community interested in prawn aquaculture and prawn health.

Further development

While this project has demonstrated that two species of prawns of commercial significance to Australia are susceptible to infection with IMNV, information on the susceptibility of prawn species other than those of the known host range (*Penaeus vannamei*, *P. stylirostris*, *P. monodon, P. esculentus* and *Fenneropenaeus merguiensis*) has not been obtained. Other species of commercial significance to Australia include the following wild-caught prawns:

Eastern king prawn (*Penaeus plebejus*)
Red spot king prawn (*Penaeus longistylus*)
Western king prawn (*Penaeus latisulcatus*)
Red-legged banana prawn (*Penaeus indicus*)
Grooved tiger prawn (*Penaeus semisulcatus*)
School prawn (*Metapenaeus macleayi*)
Blue endeavour prawn (*Metapenaeus endeavouri*)
Red endeavour prawn (*Metapenaeus ensis*)
Greasyback prawn (*Metapenaeus bennettae*)

Indeed, it is unknown whether these species of prawns are susceptible to any of the significant pathogens of prawns as listed by the OIE *Aquatic Manual*.

Extension and Adoption

The project final report will be distributed to industry, researchers and regulators with interest in prawn health.

The results of this study will be submitted for publication in a peer-reviewed scientific journal.

Project materials developed

Although not a primary objective for the project, the infectious inoculum produced during the course of the project is a valuable resource that can be used for future research on infectious myonecrosis e.g. development of positive controls for diagnostic tests, research on susceptibility of other prawn species.

References

ABARES. 2013. Australian Fisheries Statistics 2012.
http://data.daff.gov.au/data/warehouse/9aam/afstad9aamd003/2012/AustFishStats_2012_v1.0.0.pdf

Cabral da Silva SMB, Pinheiro ACAS, and Coimbra MRM. 2011 Quantitation of infectious myonecrosis virus in different tissues of naturally infected Pacific white shrimp, *Litopenaeus vannamei*, using real-time PCR with SYBR Green chemistry. *J. Virol. Meth*. **177:** 197-201.

Hasson KW, Lightner DV, Mohney LL, Redman RM, Poulos BT and White BM. 1999. Taura syndrome virus (TSV) lesion development and the disease cycle in the Pacific white shrimp *Penaeus vannamei*. *Dis. Aquat. Org.* **36:** 81-93.

Lightner DV. 2003. The penaeid shrimp viral pandemics due to IHHNV, WSSV, TSV and YHV: History in the Americas and current status. *In*: Proceedings of the 32nd Joint UJNR Aquaculture Panel Symposium, Davis and Santa Barbara, California, USA. 20pp.

Lightner DV, Pantoja CR, Poulos BT, Tang KFJ, Redman RM, Pasos de Andrade T and Bonami JR. 2004. Infectious myonecrosis: new disease in Pacific white shrimp. *Global Aquaculture Advocate* **7:** 85.

Nunes AJP, Cunha-Martins P and Vasconselos-Gesteira TC. 2004. Carcinicultura ameaçada. *Rev. Panoram. Aquic.* **83:** 37–51 (in Portuguese).

OIE. 2014. Infectious Myonecrosis.
http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.2.03_%20IMN.pdf

OIE. 2014a. Criteria for listing species as susceptible to infection with a specific pathogen. http://www.oie.int/index.php?id=171&L=0&htmfile=chapitre_criteria_species.htm

OIE. 2014b. White spot disease.
http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.2.06_WSD.pdf

Senapin S, Phewsaiya K, Briggs M and Flegel TW. 2007. Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. *Aquaculture* **266:** 32-38.

Senapin S, Phewsaiya K, Gangnonngiw W and Flegel TW. 2011. False rumours of disease outbreaks caused by infectious myonecrosis virus (IMNV) in the whiteleg shrimp in Asia. *J. Negative Results in BioMedicine* **10:** 10-14.

Tang KFJ, Pantoja CR, Poulos BT, Redman RM and Lightner DV. 2005. *In situ* hybridization demonstrates that *Litopenaeus vannamei*, *L. stylirostris* and *Penaeus monodon* are susceptible to experimental infection with infectious myonecrosis virus (IMNV). *Dis. Aquat. Org.* **63:** 261-265.

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