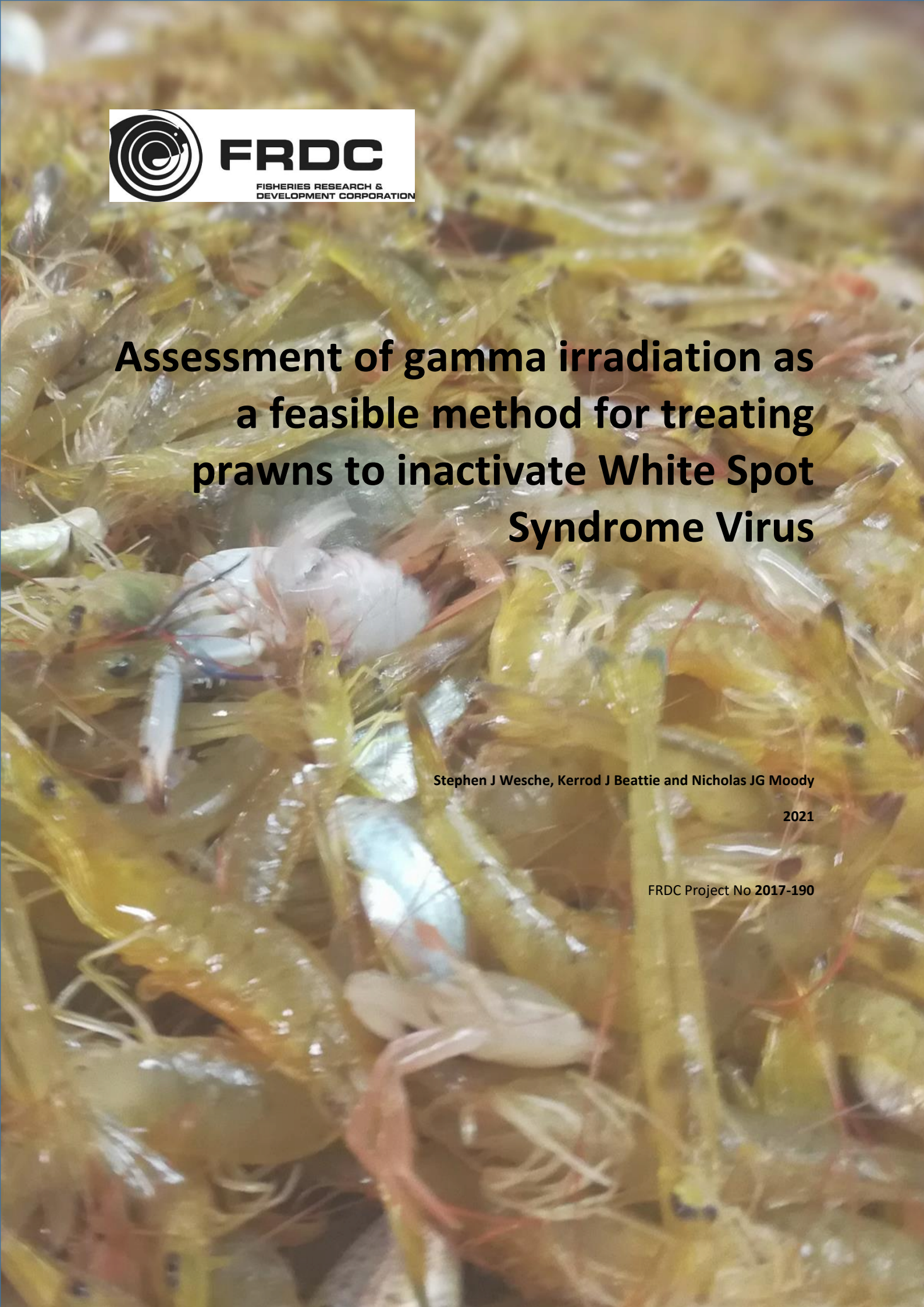

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Assessment of gamma irradiation as a feasible method for treating prawns to inactivate White Spot Syndrome Virus

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Contents

Tables

Figures

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Abbreviations

AAMI	Association for the Advancement of Medical Instrumentation
ACDP	Australian Centre for Disease Preparedness
AFDL	ACDP Fish Diseases Laboratory
AVL	Qiagen lysis buffer
bp	Base pairs
C_T	Cycle threshold
Ctrl	Control
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	Deoxyribonucleic acid
EF1	Elongation factor 1
EtOH	Ethanol
H&E	Hematoxylin and eosin
INIB	Intra-nuclear inclusion body
ISO	International Organization for Standardization
kGy	Kilogray
LAF	ACDP Large Animal Facility
NEC	Negative extraction control
NTC	No template control
PBSA	Phosphate buffered saline (Dulbecco A)
PCR	Polymerase chain reaction
POS	Positive
qPCR	Real-time polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
SPGA	Sucrose-Phosphate-Glutamate-Albumin (viral transport medium)
T4	Double-stranded DNA bacteriophage
WSD	White spot disease
WSSV	White spot syndrome virus

Executive Summary

White spot syndrome virus (WSSV) was detected in diseased farmed prawns by Biosecurity Queensland's Biological Sciences Laboratory and confirmed by the CSIRO Australian Centre for Diagnostic Preparedness (ACDP) Fish Diseases Laboratory in November/December 2016. This exotic aquatic disease incursion resulted in the infection of all prawn farms, under production, along the Logan River, Queensland. Subsequent WSSV detections in prawns and crabs in northern Moreton Bay resulted in the issuing of a Movement Control Order and subsequent WSSV biosecurity control order under the Biosecurity Act 2014, which prohibited the movement of potential carriers (e.g. polychaete worms and decapod crustaceans) from Moreton Bay. These restrictions included wild caught prawns from Moreton Bay which were distributed nationally as bait for recreational fishing. Industry established protocols to treat these prawns, for distribution as bait, using gamma irradiation to a dose of 50 kilogray (kGy). However, this treatment understandably resulted in increased costs of the final product, raising concerns that this would make imported commodity prawns a more likely source of bait for recreational fishing. Research to evaluate the effect of reduced doses of gamma irradiation (10, kGy, 15 kGy, 20 kGy and 25 kGy) on inactivation of WSSV was urgently required so this FRDC-funded, collaborative project involving Department of Agriculture and Fisheries, Queensland, CSIRO ACDP, Steritech and industry was established.

Background

Since the early 1990's ionising radiation doses of no less than 50 kGy have been required by Commonwealth Government authorities for biosecurity treatment to ensure inactivation of known pathogens of concern in certain imported commodities. Some companies have already treated bait products at 50 kGy, and while some trials have been partially successful, the treatment volumes, associated costs, and logistics in scheduling treatment cause significant logistical and financial issues. The fact that a large percentage of Australian bait prawns originate from Moreton Bay means that failure to find a viable solution to inactivate WSSV in prawns will effectively eliminate local bait supplies. A reduction in the effective gamma irradiation treatment dose, from 50 kGy to 25 kGy or lower, would significantly reduce the cost of treatment for industry to continue trade in uncooked prawns sourced from Moreton Bay.

Given the absence of information on irradiation doses for inactivation of WSSV in the Department of Agriculture (2014) review document, and the absence of research in Australia or overseas to assess alternative irradiation doses that completely inactivate WSSV, new research is urgently required to determine the minimum safe irradiation dose for inactivation of WSSV in prawns, with the results assessed by controlled challenge trials in Australia's national bio-secure animal facility at the ACDP.

Aims/objectives

The primary objective of this project was to determine whether gamma irradiation treatment of WSSV infected prawns at a dose lower than 50 kGy would inactivate the virus. The objective was further qualified by the bait wholesale industry who indicated that a reduction in treatment dose to 25 kGy or lower would be required to provide meaningful cost benefits.

Methodology

The project consisted of two trials, Trial 1 and Trial 2. The first stage of each trial involved generating WSSV-infected *P. monodon* and storing at -80°C prior to removal from the ACDP secure area where the prawns were placed in dry ice filled eskies, locked and transferred to Steritech in Brisbane for gamma irradiation to prescribed levels of gamma irradiation. For Trial 1, four eskies of WSSV-infected prawns were gamma irradiated to either 10 kGy, 15 kGy, 20 kGy or 25kGy. For Trial 2, eskies of WSSV-infected prawns were gamma irradiated to 25kGy (n=1) or 50 kGy (n=3). For both trials, a fifth esky of WSSV-infected prawns was retained with dry ice at ACDP to act as a positive infection control. Prawns generated during the first stage for each

trial were tested by CSIRO WSSV qPCR and histology to confirm infection. After gamma irradiation, prawns were returned to ACPD and stored at -80°C in the secure area.

For the second stage of each trial, live *P. monodon* were exposed to the gamma irradiated WSSV-infected prawns by injection and feeding (Trial 1) or injection and feeding or feeding only (Trial 2). For both trials, exposed live prawns were monitored daily, and moribund prawns were collected and processed for histology and WSSV qPCR testing. Prawns found dead were only sampled for WSSV qPCR testing. Prawns surviving at the end of each trial were sampled for histology and WSSV qPCR testing.

Results/key findings

Trial 1 demonstrated that gamma irradiation of WSSV-infected prawns at 10 kGy, 15 kGy, 20 kGy and 25 kGy, after exposure of *P. monodon* to this material by injection and feeding, failed to inactivate the infectious WSSV present. While the different doses of gamma irradiation used did delay the onset of mortalities, compared to exposure to untreated WSSV-infected P*. monodon*, cumulative mortalities of >90% occurred in all treatment groups. Negative control cumulative mortality was 0%. Infection with WSSV in exposed prawns was confirmed by positive WSSV qPCR test results, with C_T values in all prawns similar to positive infection control prawns (i.e. prawns exposed to WSSV-infected prawns that had not been gamma irradiated). Pathology observed in histological sections confirmed the white spot disease (WSD) in all exposed prawns, including those exposed to material gamma irradiated to 25 kGy.

Based on cumulative mortalities of <4%, and lack of histological evidence of WSD in prawns sampled at the end of Trial 2, gamma irradiation to 50 kGy inactivated the infectious WSSV present. After exposure by feeding, based on similar cumulative mortality of <4% and absence of histological lesions indicative of WSD, gamma irradiation at 25 kGy also inactivated the WSSV in the infected prawns.

Unfortunately, unexpected positive WSSV qPCR test results were observed in prawns that had survived until the end of the 28-day Trial 2, where cumulative mortality was <4% in all prawns exposed to gamma irradiated WSSV-infected prawns. These positive results may be due to low-level subclinical infection, residual WSSV circulating in the prawns (after injection and/or ingestion of non-infectious gamma irradiated WSSV-infected prawns) or residual WSSV DNA in the water in the tanks due to the gamma irradiated WSSV-infected prawns. It is considered most likely these WSSV qPCR positive test results were due to contamination with non-viable WSSV nucleic acid from the commercial prawn feed used.

Implications for relevant stakeholders

Based on results from Trial 1 and some uncertainty relating to the results of Trial 2 (unexpected WSSV positive test results in prawns surviving infection), wild prawns harvested within the WSSV movement restriction area for packaging and sale as fresh/frozen fishing bait should continue to be treated with gamma irradiation at a dose of 50 kGy.

Recommendations

Due to the unexpected detection of WSSV nucleic acid in experimental animals during Trial 2, and the suggestion that these qPCR test results are due to contamination from non-viable WSSV nucleic acid in feed, Trial 2 should be repeated after a source of WSSV-negative feed has been identified. If this is not possible, consideration should be given to modifying the experimental design to include testing of samples less susceptible to surface contamination, such as hemolymph.

Keywords

White spot syndrome virus, WSSV, gamma irradiation, pathogenesis, real-time PCR, qPCR.

Introduction

On 1 December 2016 the internationally notifiable pathogen White Spot Syndrome Virus (WSSV) was confirmed in prawn farms on the Logan River in Moreton Bay Queensland. Subsequent investigations by Biosecurity Queensland found that low numbers of wild prawns and crabs caught in the Logan River and Northern Moreton Bay were also positive for WSSV, however recent delimitation surveys have not found the virus elsewhere in Queensland waters.

The presence of WSSV in prawn farms and the environment has required a response by Biosecurity Queensland which has included biosecurity measures such as the implementation of Movement Control Orders for potential WSSV carrier species. On 16 July 2017 the Movement Control Order was replaced by The Biosecurity (White Spot Syndrome Virus) Amendment Regulation 2017. The amendment regulation established biosecurity zones which include Moreton Bay and its catchments to contain and control the WSSV infection and prevent its spread to uninfected areas by restricting where some product (prawns, yabbies, worms) can be moved to, used or sold, but allow all activities within the area to continue.

Impacts on commercial fishers is difficult to formally quantify however there is sufficient evidence that fishers who operate in the Moreton Bay area whose catch is prawns, either for human consumption or bait have been disadvantaged because of the movement restrictions imposed for carrier species (prawns, yabbies, worms). Further, there are significant impacts on the smaller bait harvesting operations for yabbies, beach and blood worms.

Current restrictions mean that uncooked WSSV carrier species are not permitted to be moved from within the restricted area to outside of this area unless they have a permit to do so. Despite the fact that cooking can inactivate WSSV, cooking is not considered to provide an acceptable end product for the commercial bait prawn or worm markets. Recently opportunities have been explored to find alternate treatment methods such as gamma irradiation, rendering the WSSV inactive, and therefore present minimal risk to moving these products from within the restricted area. Gamma irradiation kills microorganisms by damaging and/or denaturing their genetic material.

Since the early 1980's gamma irradiation has been accepted by the Federal Department of Agriculture as a biosecurity treatment for products of animal origin. Since the early 1990's ionising radiation doses (quoted as kilogray (kGy)) of no less than 50 kGy have been required by Federal authorities for biosecurity treatment to ensure inactivation of all known pathogens of concern in certain imported commodities.

Some companies have already treated bait products at 50kGy and while some trials have been partially successful the treatment volumes, associated costs, and logistics in scheduling treatment cause significant issues. The fact that a large percentage of Australian bait prawns originate from Moreton Bay means that failure to find a viable solution to inactivate WSSV in prawns will effectively eliminate local bait supplies, potentially driving consumers to increase their use of imported uncooked prawns from supermarkets as bait.

Achieving the mandated dose rate of 50 kGy for prawns harvested from the White Spot Disease control zone in SE QLD is proving to be problematic and industry are requesting a reduction in the permitted irradiation dose rate for prawns sourced from Moreton Bay to somewhere between 15 and 25 kGy. A reduction in treatment dose to 25 kGy or lower, if successful, would significantly reduce the cost of treatment for industry to continue trade in uncooked prawns sourced from Moreton Bay.

There appears to be only three scientific papers relevant to the subject of using gamma irradiation for inactivation of WSSV (Heidareh et al., 2014, Kakoolaki et al., 2015, Motamedi-Sedeh et al., 2017). The studies were conducted with the aim of determining the doses required to inactivate the Iranian isolate of WSSV with gamma irradiation for the purposes of vaccinating cultured prawns. While the listed scientific papers suggest gamma irradiation at a dose of around 13-15 kGy is effective at inactivating WSSV, their experimental

protocols and results are not clearly transferable. In these studies aliquots of free virus were exposed to different gamma irradiation doses. Irradiation of WSSV inside infected host tissues was not performed, and data on whether the surviving prawns were infected with WSSV were not presented. None of these papers are therefore directly applicable to the situation relating to irradiation treatment of WSSV in-situ inside infected prawns.

Given the absence of information on irradiation doses for inactivation of WSSV in the Department of Agriculture (2014) review document, and the absence of research in Australia or overseas to validate alternative irradiation doses that completely inactivate WSSV, new research is urgently required to determine the minimum safe irradiation dose for inactivation of WSSV in prawns with the results verified by controlled challenge trials in a bio-secure facility.

Objectives

1. To determine minimal effective dose of gamma irradiation to render WSSV non-infectious in prawns.

1. Experimental Trial 1: Assessment of gamma irradiation at 10, 15, 20 and 25 kGy

The aims of Experimental Trial 1 were:

1. To amplify WSSV in *P. monodon* and expose to gamma irradiation at 10, 15, 20 and 25 kGy.

2. To determine the effect of the different doses of gamma irradiation on the infectiousness of WSSV in the irradiated *P. monodon*.

1.1. Generation of WSSV-infected *P. monodon* for gamma irradiation

1.1.1. Methods

1.1.1.1 Experimental prawns

Prawns were sourced from a commercial prawn culture facility. On arrival prawns (~7 to 11cm total length) were transferred to the secure ACDP Large Animal Facility (LAF) and placed in square polypropylene tanks containing approximately 80 L seawater, a canister filter and air stones. Prawns were distributed across two rooms (for negative controls and WSSV inoculation). Each tank held 21 to 25 prawns with water temperature maintained at 28.8 $\pm$ 0.6°C for WSSV inoculated prawns and 25 $\pm$ 1°C for negative control prawns. Seven replicate tanks were used in the room where prawns were exposed to WSSV. At least one tank in each room was fitted with a data-logger to measure temperature. Animals were monitored and fed daily with commercial prawn feed. Water exchanges (30%-50%) were undertaken every 2 days.

1.1.1.2 Preparation of inoculum

Material from the index case received at ACDP from Biosecurity Queensland on 30 November 2016 for confirmation of detection of WSSV in IP1 was used as the inoculum. These samples consisted of 10 x gill homogenates in SPGA (16-03907) and 10 x carapace homogenates in SPGA (16-03908). Approximately, 500 µl was removed from each gill homogenate tube and 75 µl from each carapace homogenate tube and these were pooled. After clarification at 3000 rpm for 5 minutes the supernatant was removed and added to 20 ml PBSA to produce the inoculum. A 200 µl aliquot was stored at -80°C for WSSV qPCR testing.

1.1.1.3 Inoculation of prawns, sampling, and sample processing

Prior to inoculation, 10 negative control animals were sacrificed and fixed in 80% (v/v) ethanol (EtOH) for molecular testing to exclude WSSV. For inoculation, prawns were caught with a net and held firmly to avoid tail-flick response. Each was inoculated with 100 µl of the inoculum into the second abdominal segment using a 26-gauge needle and placed back in the tank. Negative control animals were sham-inoculated by insertion of a 26-gauge needle.

For prawns found dead after inoculation during the trial, three pleopods were removed and placed into individually labelled 2 ml tubes containing 80% (v/v) EtOH. The rest of the prawn was pooled with a further 7 to 10 dead prawns in a labelled zip-lock bag in a plastic container and stored at -80°C. At the end of the trial, surviving prawns were bled from the ventral sinus using a 26-gauge needle into a syringe containing 10% sodium citrate.

A sub-sample from one pleopod from each prawn was added to a bead beating tube containing 600 µl AVL buffer and homogenised using a FastPrep-24 bead-beater (MP Biomedicals). Homogenates were clarified by centrifugation at 10 000 × g for 5 min. Total nucleic acid was extracted from 50 µl of the supernatant using

the MagMAX-96™ Viral RNA Isolation Kit on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Life Technologies). Prior to extraction, 5 µl of T4 DNA phage/sample was added to the MagMAX lysis buffer as an internal control to assess inhibition of the real-time PCR (Moody et al., 2021). Nucleic acid was eluted in 50 µl elution buffer and stored at -80°C prior to testing.

1.1.1.4 Molecular Testing for WSSV

The WSSV real-time tests used in this report depended on the sample to be tested (e.g. inoculum, WSSV-infected prawn, feed). Some samples were tested with only the CSIRO WSSV qPCR and others tested with both the CSIRO WSSV qPCR and OIE WSSV qPCR. The CSIRO WSSV qPCR uses the primers and probe described by East et al., (2004) and the OIE WSSV qPCR uses the primers and probe described by Durand and Lightner (2002) with the following reaction and cycling conditions; each 25 µl reaction mix contained 2 µl extracted nucleic acid template, 12.5 µl TaqMan Universal PCR Master Mix (Life Technologies), a final concentration of 900 nM for each primer, 250 nM for the probe and molecular-grade water. The qPCR assays are performed in a 7500 Fast Real-Time PCR System or QuantStudio 5 Real-Time PCR System (Life Technologies) and analysed with the 7500 software or QuantStudio design and analysis software respectively. PCR amplifications are programmed as follows: 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. A threshold of 0.1 is used to determine C_T value. Nucleic acids were also tested, in singlicate using the T4 DNA Phage internal control qPCR to detect and PCR inhibition (Moody et al., 2021). Two plasmid positive controls, with different expected C_T values, a negative extraction control (NEC) and a no template control (NTC) were included on each plate.

For the WSSV-infected prawns, extracted nucleic acids were tested at neat and diluted 1/10, in duplicate using the CSIRO WSSV qPCR, to confirm the presence of WSSV and determine if the level of infection was suitable for the material's intended purpose.

1.1.1.5 Gamma irradiation of WSSV-infected P. monodon

The dosimeters (Harwell Red 4034 dosimeter, Harwell Dosimeters Ltd., Oxfordshire, United Kingdom) to be used during irradiation trials to measure the absorbed doses delivered to the samples are adversely affected by temperature extremes. Therefore, prior to the irradiation of the samples, a performance qualification was undertaken for the purpose of validating the inference of the absorbed dose delivered to the samples from the absorbed dose delivered to a dosimeter placed in a reference position on the exterior of the esky (i.e. where the dosimeter is not affected by the dry ice). The performance qualification also provides guidance on the exposure time required to achieve the target dose for each sample.

The performance qualification practice follows that outlined in ISO 11137-3:2017 and utilises a Techni Ice esky the same as that to be used for the trials except that it was filled with a salt and stock feed mix (having a similar bulk density as dry ice) instead of dry ice. The statistical methodology used to infer the absorbed dose delivered to the samples from the measured reference dosimeter dose follows AAMI TIR 29:2012 A.4 Procedure 2.

The absorbed doses measurements are performed using Harwell Red 4034 dosimeters, a Cary-60 UV-Vis Spectrophotometer and a Peacock DG-205 thickness gauge.

WSSV-infected prawns were removed from the secure area of ACDP and stored at -80°C. Prior to transport, five replicate 80L Techniice eskies were prepared. Each esky contained four pots of WSSV-infected prawns and dry ice. Four eskies were transported to Steritech for gamma irradiation at 10, 15, 20 and 25 kGy with the fifth esky retained at ACDP as a positive infection control. Each esky was labelled with the target dose.

After receipt of the eskies containing the infected samples at Steritech, a dosimeter was placed on each esky in the reference position. Each esky was then topped-up with dry ice to bring its total weight up to 65 kg (the same weight as the esky filled with the salt and stock feed mix used for the performance qualification) and

then irradiated in Steritech's IR-217 Cobalt-60 gamma irradiator. Each esky was given a radiation exposure time estimated to deliver a maximum absorbed dose to the samples as close to but not exceeding the target dose. Following irradiation, the reference dosimeter is removed, its absorbed dose measured, and the absorbed doses delivered to the samples are calculated.

The irradiated eskies were then transported back to ACDP. Upon return at ACDP, the prawns were removed from the eskies and returned to -80°C storage in the secure area.

1.1.2 Results

1.1.2.1 Exposure of P. monodon *to WSSV*

One day post-exposure, two prawns were found dead and these prawns were discarded. Two days post-exposure 55 prawns were found dead and three days post-exposure 97 prawns were found dead. Haemolymph was bled from the surviving 9 prawns and the experiment was terminated. Cumulative mortality curves for each replicate tank were very consistent and are depicted in Figure 1. Total mortality across the 7 replicate tanks ranged from 87.5% to 100% when the trial was terminated. There were no mortalities observed in negative control prawns (data not shown). A total of 140 WSSV-infected prawns stored for gamma irradiation.

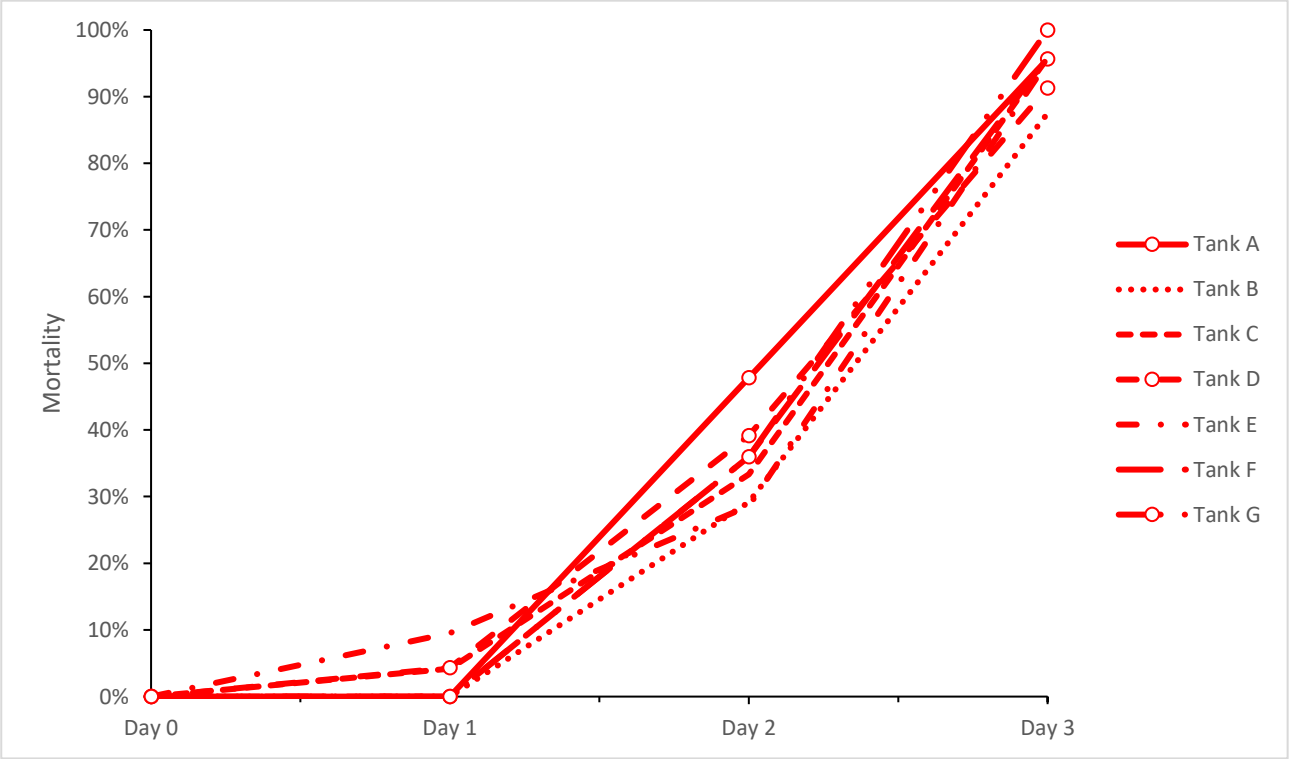


Figure 1. Cumulative mortality after exposure to WSSV by inoculation

1.1.2.2 Molecular testing for WSSV

Result of testing the nucleic acid extracts using the CSIRO WSS qPCR and T4 DNA Phage qPCR are presented in Table 1 to Table 4. The results highlight the need to use an internal control when testing prawn tissue samples due to the presence of PCR inhibitors, whether these are compounds in the tissue or excessive amounts of extracted host nucleic acid. Fifty percent of the T4 house-keeping gene qPCR results for samples tested neat are outside the acceptable range (Table 3) which indicates the WSSV qPCR results for samples tested neat are unreliable (Table 1). When these samples were tested after 1/10 dilution, all T4 house-

keeping gene qPCR results for samples tested were within the acceptable range (Table 4) indicating that the corresponding WSSV qPCR results for samples tested after 1/10 dilution are reliable (Table 2).

Table 1. CSIRO WSSV qPCR Result Summary – Neat Samples

No. Positive	No. Negative	No. Indeterminate	Mean C_T Range
130	7	3	13.56 - 38.33

Table 2. CSIRO WSSV qPCR Result Summary – Diluted (1/10) Samples

No. Positive	No. Negative	No. Indeterminate	Mean C_T Range
140	0	0	16.41 - 23.82

Table 3. T4 DNA Phage Std. qPCR Result Summary – Neat Samples

No. Positive	No. Negative	No. of Pos within acceptance range	No. of Pos outside acceptance range	CT Range
70	70	10	60	29.41 - 44.97

Table 4. T4 DNA Phage Std. qPCR Result Summary – Diluted (1/10) Samples

No. Positive	No. Negative	No. of Pos within acceptance range	No. of Pos outside acceptance range	CT Range
140	0	140	0	31.91 - 34.27

Valid results were obtained when nucleic acid extracts were tested after 1/10 dilution. The WSSV qPCR C_T values (16.41 - 23.82) observed are typical of those obtained when testing diseased prawns during the WSSV Emergency Response in 2016/17 (Figure 2).

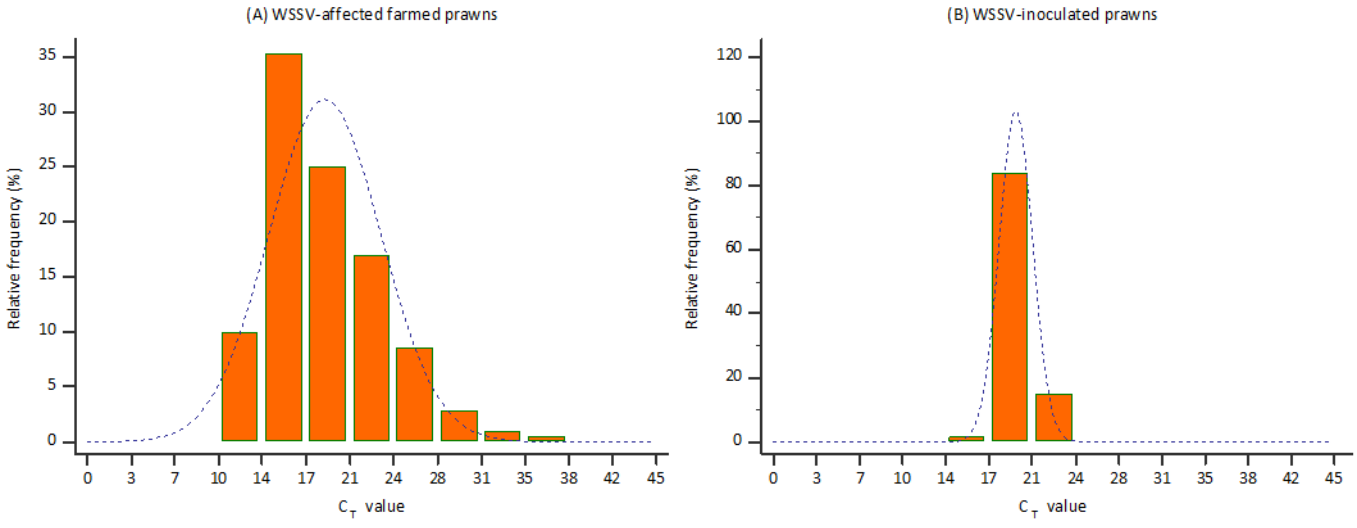


Figure 2. Frequency distribution of WSSV C_T values obtained after testing prawns from (A) index ponds from prawn farms in the Logan River in 2016/17 and (B) the infectivity trials described in this section of the report.

1.1.2.3 Gamma irradiation of WSSV-infected P. monodon

Cold chain was maintained throughout the transport and gamma irradiation process of the prawns. Cold chain was assessed by holding the fifth esky of WSSV-infected prawns in the non-secure area of ACDP, while the other four eskies were sent to Steritech and returned after gamma irradiation. On return of the eskies from Steritech, it was noted that dry ice was present in all five eskies and all prawns were frozen.

The results of the performance qualification undertaken prior to the irradiation of the WSSV-infected samples demonstrate that the maximum absorbed dose delivered to samples inside an irradiated esky would be 69.0% of the absorbed dose delivered to the dosimeter placed in the reference position on the outside of the esky. Therefore, the absorbed dose delivered to the samples in each esky are calculated by multiplying the measured reference dosimeter dose by 69%. Table 5 summarises the absorbed doses delivered to each irradiated esky.

Table 5. Absorbed Dose Result Summary

Esky/Target Dose	Measured Reference Dose	Maximum Dose Absorbed by Samples
10 kGy	14.4 kGy	9.9 kGy
15 kGy	21.5 kGy	14.8 kGy
20 kGy	28.8 kGy	19.9 kGy
25 kGy	36.3 kGy	25.0 kGy

The experimental infection generated 140 WSV-infected *P. monodon* with WSSV qPCR C_T values representative of those seen during the WSSV outbreak in farmed prawns in the Logan River in 2017/18. These prawns were successfully transported to Steritech and gamma irradiated at the required dose and returned to ACDP for future bioassay work.

1.2. Bioassay of WSSV-infected material after gamma irradiation at 10, 15, 20 and 25 kGy

1.2.1 Methods

1.2.1.1 Experimental prawns

Prawns were sourced from a commercial hatchery. On arrival prawns (~7 to 8cm total length) were transferred to the secure ACDP LAF and placed in round polypropylene tanks containing approximately 80L seawater, a canister filter and air stones. Prawns were distributed across 6 rooms (negative controls, 10 kGy, 15 kGy, 20 kGy, 25 kGy and WSSV positive infection control). Each tank held 30 to 35 prawns with water temperature maintained at 29 $\pm$ 1°C. Three replicate tanks were used in the rooms where prawns were exposed to WSSV. Two replicate tanks were set-up in a separate room with uninfected prawns as negative controls. At least one tank in each room was fitted with a data-logger to measure temperature. Animals were monitored and fed daily with commercial prawn feed supplied with the prawns and 30% water exchanges were undertaken every 1 to 2 days.

1.2.1.2. Preparation of inoculum

Fours pots of prawns which had been infected with WSSV were in each of five 80L eskies (four eskies were sent to Steritech for gamma irradiation and one was held at ACDP as untreated WSSV-positive infection controls). To prepare each of the inoculums, one prawn from each of the four pots was thawed in a Class II Biological Safety Cabinet and the gills from one side removed with a sterile scalpel. The gills from the four

prawns were homogenised into a paste using a chilled mortar and pestle and resuspended in 25ml PBSA. After centrifugation at 3000 rpm for 10 minutes at 18°C the supernatant was filtered through 1.2 µm and 0.45 µm syringe filters. A 1ml aliquot was stored at -80°C for CSIRO WSSV qPCR testing and the remainder was immediately transferred to the ACDP LAF to be used to inoculate the prawns. Three prawns from the remaining material were put into multiple ziplock bags and stored at -80°C to be fed to the prawns daily during the trial.

1.2.1.3 Inoculation and feeding of prawns and sampling

Prior to inoculation, 10 negative control animals were sacrificed. Pleopods were fixed in 80% (v/v) EtOH for molecular testing and the head was fixed in Davidson's Fixative for histological analysis. For inoculation, prawns were caught with a net and held firmly to avoid tail-flick response. Initially, each prawn was inoculated with 100 µl of the inoculum into the second abdominal segment using a 26-gauge needle and placed back in the tank. However, shortly after inoculation with 100 µl a number of the prawns died so the volume was reduced to 30 to 40 µl. This reduced volume led to a significant reduction in the number of prawns subsequently dying. Negative control animals were sham-inoculated by insertion of a 26-gauge needle.

From Day 2 the remaining gamma irradiated prawns (from the same gamma irradiation dose as the inoculum) were fed to the prawns in each of the three tanks that had been inoculated on Day 1. This feeding of the irradiated prawns was undertaken to ensure that prawns were exposed to all of the irradiated material (in the event that no mortalities were observed). For each tank, three frozen prawns in a zip-lock bag were cut in half longitudinally with a sterile scalpel, then sliced into small pieces and fed to the prawns in the tanks. There were sufficient gamma irradiated prawns to use as feed for each treatment for the duration of the trial. Negative control prawns were fed commercial prawn feed.

Prawns were observed daily. When prawns were found dead, two pleopods were removed and placed into individually labelled 2 ml O-ring, screw-cap tubes containing 80% (v/v) EtOH. The rest of the prawn was pooled with other dead prawns in a labelled zip-lock bag in a plastic container and stored at -80°C. For observed moribund prawns, two pleopods were removed and placed into individually labelled 2 ml O-ring, screw-cap tubes containing 80% (v/v) EtOH. The head was then cut in half longitudinally with both halves put in Davidson's Fixative in a labelled 50 ml specimen container. Alternatively, the head was injected with Davidson's Fixative and placed in a labelled 50 ml specimen container containing fixative. After 24 hours tissues were transferred to 70% (v/v) EtOH. Tissues for histology were processed for paraffin-embedding, sectioning and H&E staining using standard procedures. Sections were examined using a compound light microscope or digitally scanned using a whole slide imaging system and viewed on a computer.

1.2.1.4 Processing and molecular testing for WSSV

A sub-sample from one pleopod from each prawn was added to a bead beating tube containing 600 µl PBSA and homogenised using a FastPrep-24 bead-beater (MP Biomedicals). Homogenates were clarified by centrifugation at 10 000 × g for 5 min. Total nucleic acid was extracted from 50 µl of the supernatant using the MagMAX-96™ Viral RNA Isolation Kit on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Life Technologies). Prior to extraction T4 DNA phage (5 µl per sample) was added to the MagMAX lysis buffer as an internal control to assess any inhibition of the real-time PCR. Nucleic acid was eluted in 50 µl elution buffer and stored at -80°C prior to testing. Extracted nucleic acids were tested at neat and diluted 1/10 using the CSIRO WSSV qPCR to confirm the presence of WSSV as described above (Section 1.1.4).

1.2.2 Results and Discussion

1.2.2.1 Preparation of inoculum and exposure of P. monodon *to WSSV*

Result of testing the nucleic acid extracted from the inoculums using the CSIRO WSSV qPCR and OIE WSSV qPCR assays is presented in Table 1. The narrow C_T range (13.36-14.44 and 14.39-15.56, respectively) demonstrates the uniformity of the different inoculum preparations but is not a measure of the level of infectivity. As the effect of the gamma irradiation in the WSSV was completely unknown, the inoculums were not tested by qPCR until after inoculation of the prawns to reduce any reduction in infectious virus, due to extended holding at 4°C or freeze thawing if stored at -80°C.

Table 6. CSIRO WSSV qPCR and OIE WSSV qPCR results for each inoculum used.

Inoculum	CSIRO WSSV qPCR Mean C_T value	OIE WSSV qPCR Mean C_T value
WSSV - untreated	14.44	15.52
WSSV - 10 kGy	14.33	15.56
WSSV – 15 kGy	13.36	14.39
WSSV - 20 kGy	13.54	14.67
WSSV - 25 kGy	13.63	14.67

No mortalities were observed in negative control prawns. A high number of mortalities were observed 1-day post infection (dpi) in prawns exposed to the untreated WSSV-infected prawns. In general, mortalities occurring 1 dpi are excluded from the analyses as they are usually due to inoculum toxicity or the process of injecting the animals. However, as the highest number of mortalities (117/190; 62%) were observed in prawns injected with the untreated WSSV, a number of these may actually be due to the pathogenicity of the virus injected. The inoculum had a mean C_T value of 14.44 and high-level mortalities occurring 1 dpi have been observed previously when a WSSV qPCR positive prawn homogenate had been injected into prawns (M. Crane, pers. comm.). The prawns injected with the inoculum from the 20kGy sustained approximately 50% mortalities shortly after injection. However, this was most likely due to injection with 100µL. When the volume was reduced to 30 to 40 µl, mortalities were not observed although the initial mortalities resulted in reduced animal numbers across the tanks inoculated with this material.

Excluding mortalities occurring on 1 dpi, mortalities of 90-100% were observed across all treatment groups (Figure 3). All prawns exposed to the untreated WSSV (POS group) and WSSV treated with 10 kGy and 15 kGy died. Mortalities of 90% and 91% were observed in prawns exposed to WSSV treated with 20 kGy and 25 kGy respectively and the experiment was terminated at this point. Only 4 prawns remained across the three tanks for 20 kGy and 5 prawns in the 25 kGy treatment groups when the trial was terminated. As significant mortalities had been observed, these prawns were euthanased and sampled for molecular testing and histology. Compared to prawns inoculated with the untreated WSSV material, the time to total mortality was delayed depending on the dose of gamma irradiation. No gross signs of white spot disease were observed on any prawns.

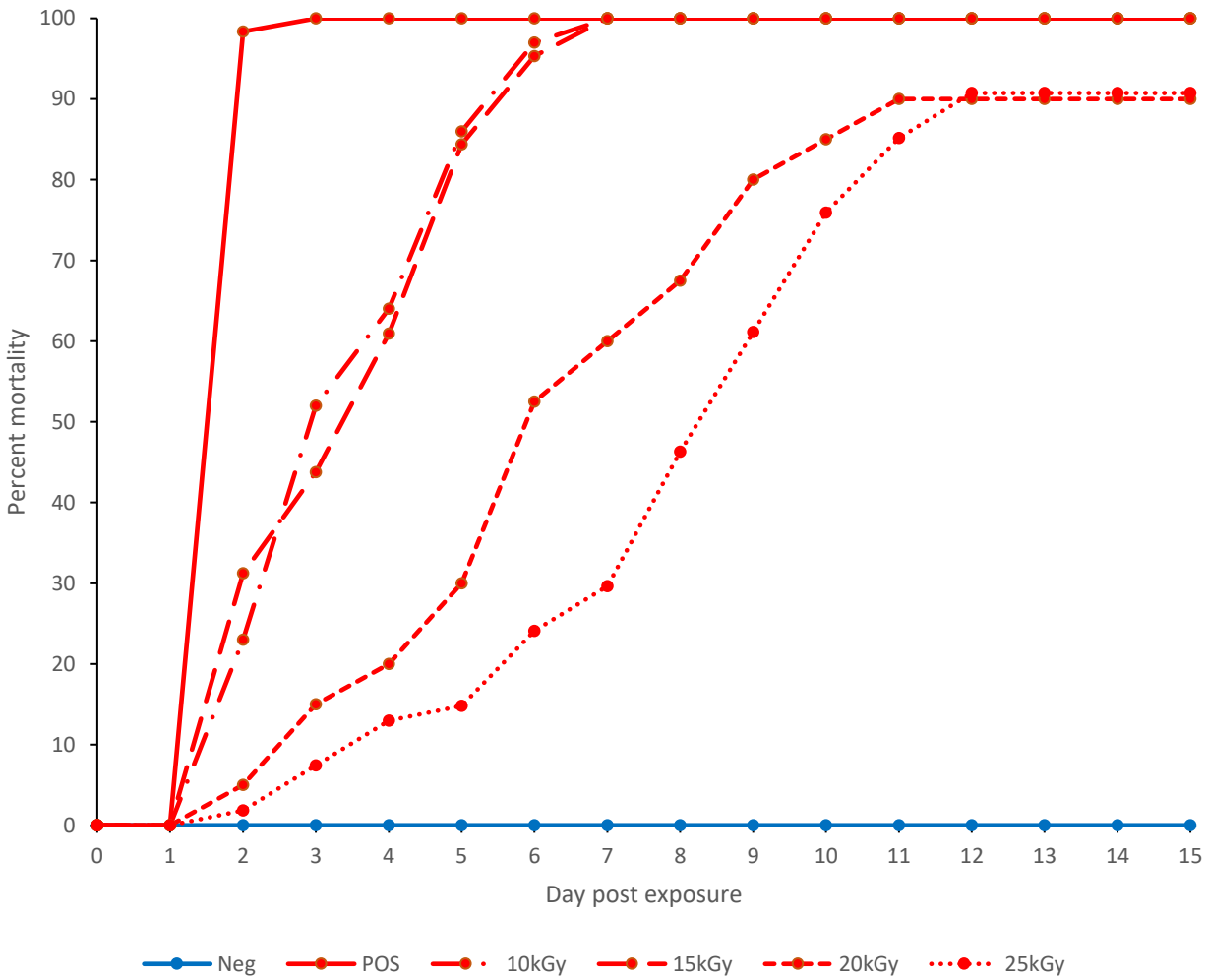


Figure 3. Cumulative mortality, across combined replicates, in *P. monodon*, after exposure to WSSV irradiated with different doses of gamma irradiation.

Mortalities were observed sooner in prawns exposed to the lower doses of 10 kGy and 15 kGy with the onset of mortalities delayed longest in prawns exposed to the highest dose of 25 kGy. However, as 90% mortality was observed after 12 days in this group, it is fair to conclude that based on these mortalities, that while the gamma irradiation had reduced the infectious dose in the inoculum, it had not inactivated all WSSV present. Mortality curves between replicates for each treatment (Figures 4 to 7) were similar, although the onset of mortalities was delayed in one tank (Tank A) in the 20 kGy treatment group (Figure 6). This was the first tank of prawns inoculated in the room and highlights the need to have appropriate replication for experimental trials.

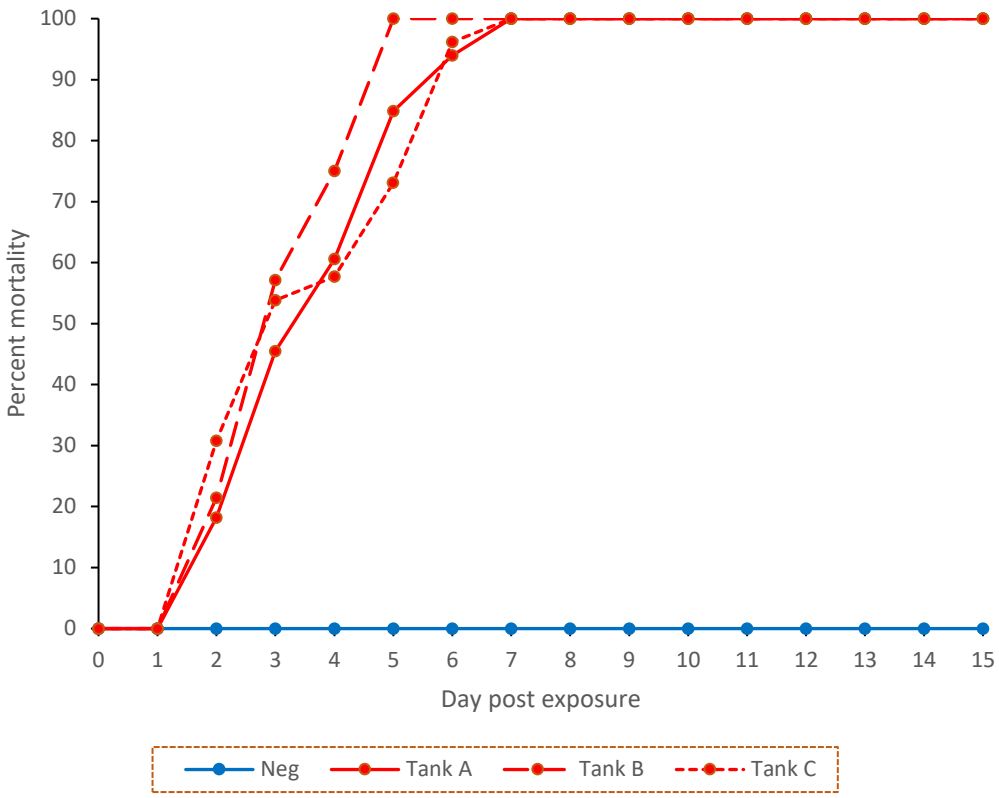


Figure 4. Cumulative mortality in replicate tanks of *P. monodon*, after exposure to WSSV-infected prawns, gamma irradiated to 10 kGy.

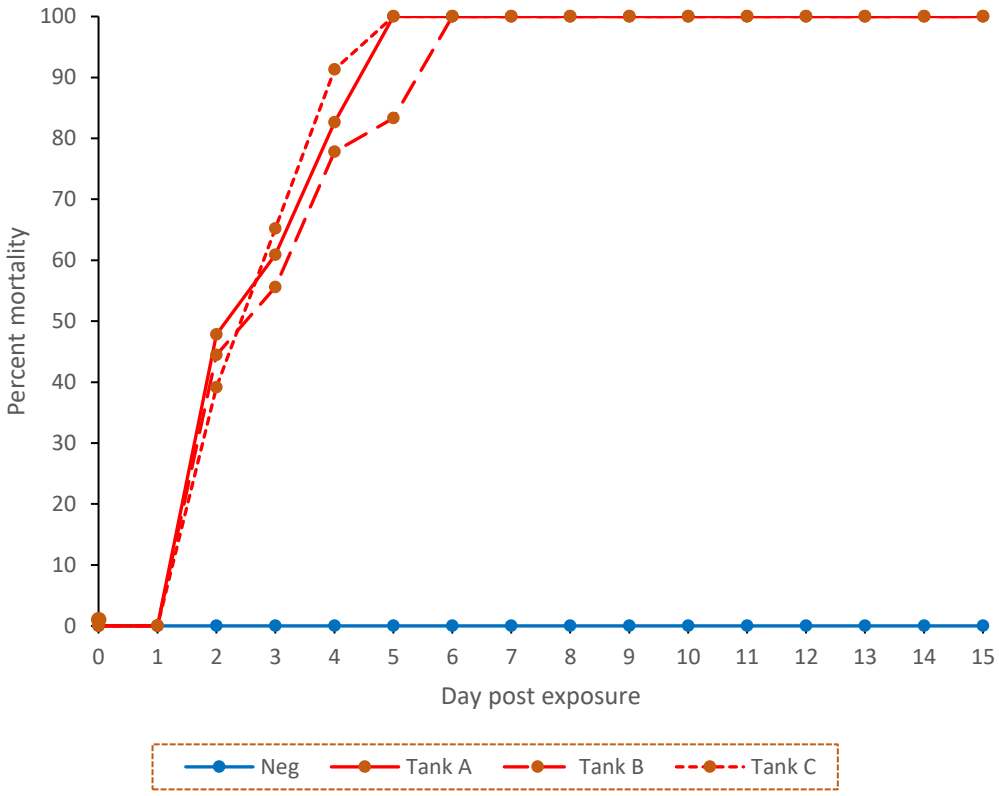


Figure 5. Cumulative mortality in replicate tanks of *P. monodon*, after exposure to WSSV-infected prawns, gamma irradiated to 15 kGy

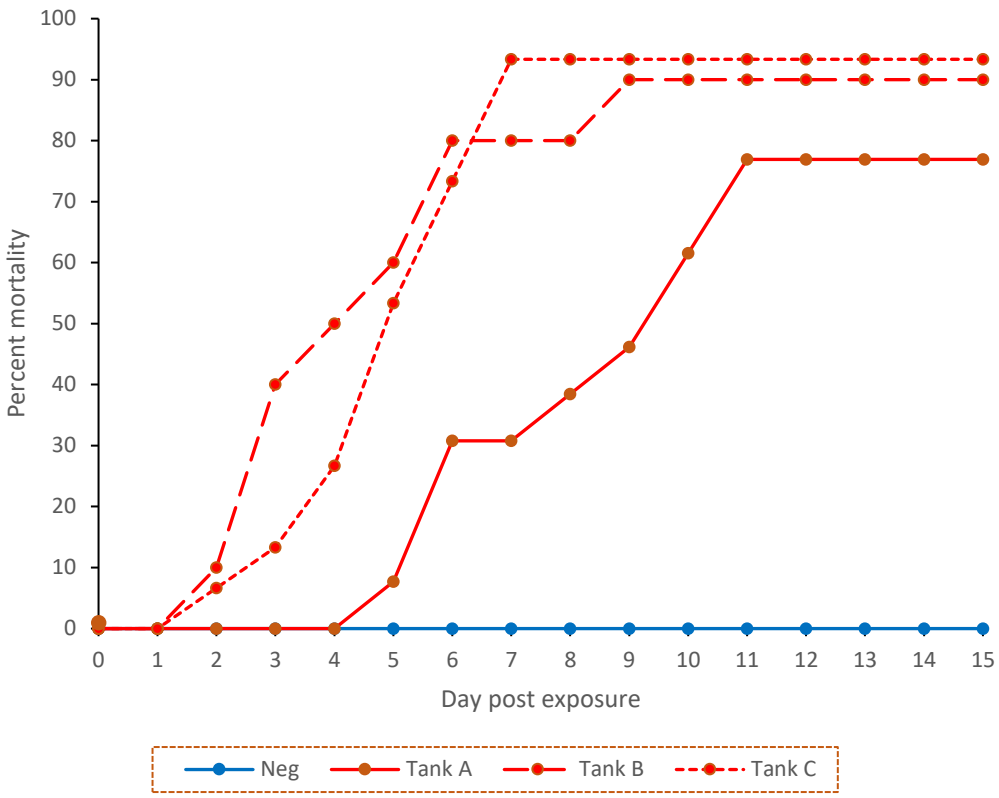


Figure 6. Cumulative mortality in replicate tanks of *P. monodon*, after exposure to WSSV-infected prawns, gamma irradiated to 20 kGy

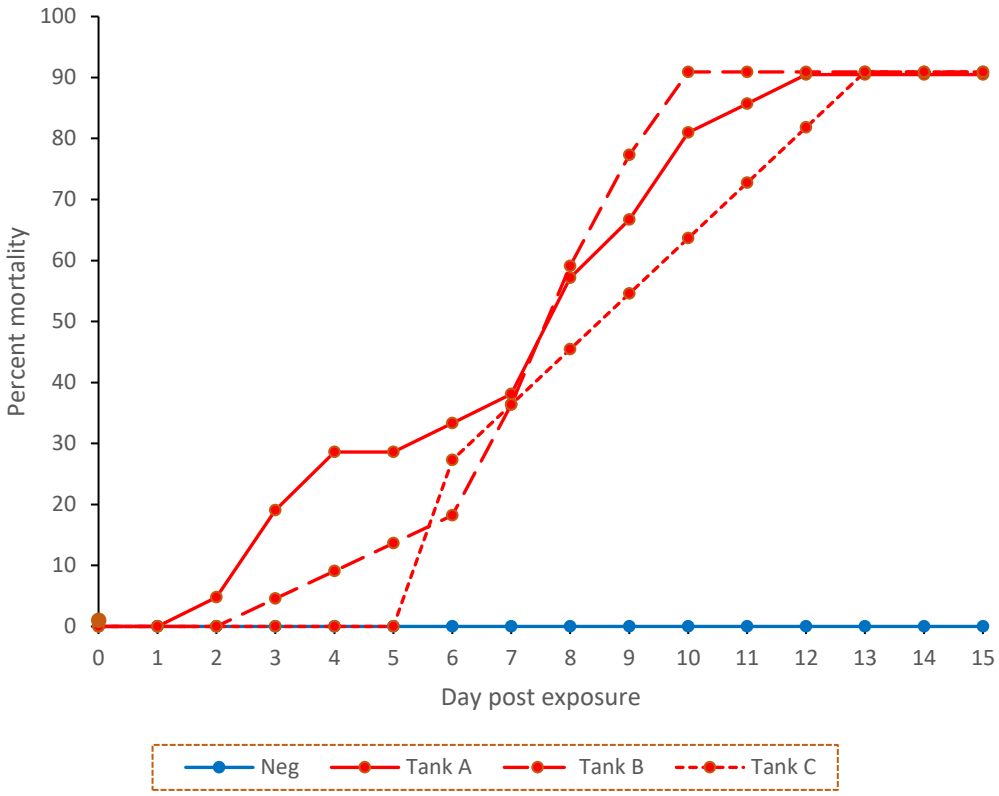


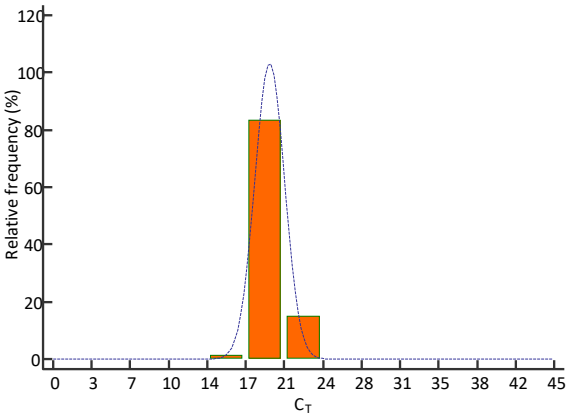
Figure 7. Cumulative mortality in replicate tanks of *P. monodon*, after exposure to WSSV-infected prawns, gamma irradiated to 25 kGy

1.2.2.2 Molecular testing of P. monodon for WSSV after exposure to WSSV-infected gamma irradiated prawns.

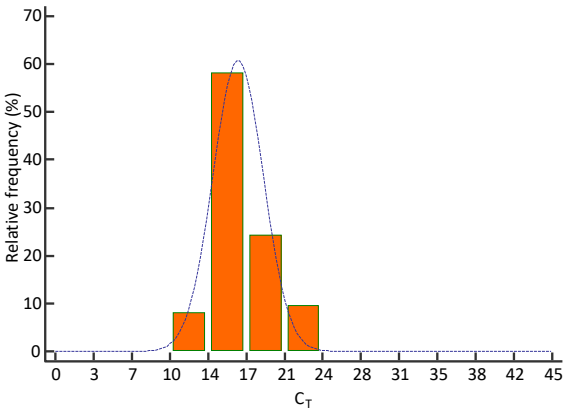
Pleopods from each dead or moribund prawn were tested by the CSIRO WSSV qPCR. All prawns from all treatments, excluding negative controls, tested positive (Table 7). The prawns exposed to untreated WSSV and the two lower doses of gamma irradiation (10 kGy and 15 kGy) had C_T values in a narrower and lower range. The frequency distributions of C_T values for all gamma irradiation treatment groups (Figure 8) are within the range expected for clinically diseased prawns. The wider variation seen with the prawns exposed to WSSV irradiated at 20 kGy and 25 kGy is most likely due to a reduced dose of infectious virus in this material, caused by the gamma irradiation. This has resulted in a less acute infection than in prawns exposed to WSSV gamma irradiated with 10 kGy and 15 kGy, which is also supported by the cumulative mortality curve data. One important point to note is, although mortalities were delayed with prawns exposed to WSSV irradiated to 25 kGy, one sample from this population of animals produced the lowest C_T value of 12.71 (Table 7). This is lower than the C_T value of the inoculum, which was 13.63, and is indicative of the presence of infectious, replicating WSSV. All negative control samples tested negative.

Table 7. Summary CSIRO WSSV qPCR test results

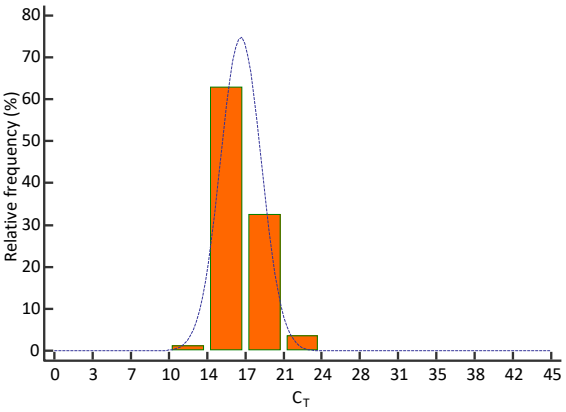
Treatment of inoculum	CSIRO WSSV qPCR Test Data			CSIRO WSSV qPCR C_T values				
	Number Tested	Number Negative	Number Positive	Lowest	Highest	Median	Mean	SD
WSSV POS	74	0	74	12.76	22.73	16.15	16.56	2.27
10 kGy	86	0	86	13.72	21.16	16.59	16.90	1.85
15 kGy	64	0	64	13.28	19.70	15.64	15.98	1.64
20 kGy	38	0	38	14.05	27.54	20.42	20.39	3.92
25 kGy	59	0	59	12.71	26.94	18.65	18.97	3.40
Negative controls	20	20	0	-ve	-ve	-ve	-ve	-ve



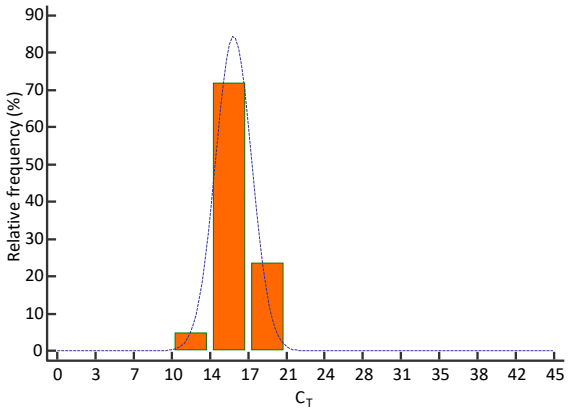
(A) Amplification of WSSV to generate the infected prawns for gamma irradiation.



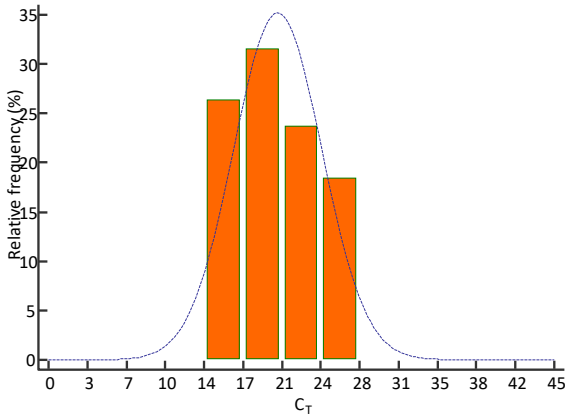
(B) WSSV Positive infection control (untreated WSSV-infected prawns)



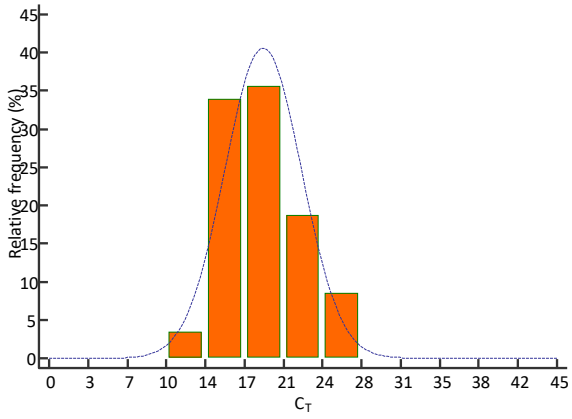
(C) After exposure to WSSV-infected prawns after gamma irradiation to 10 kGy



(D) After exposure to WSSV-infected prawns after gamma irradiation to 15 kGy



(E) After exposure to WSSV-infected prawns after gamma irradiation to 20 kGy



(F) After exposure to WSSV-infected prawns after gamma irradiation to to 25 kGy

Figure 8. Frequency distribution of WSSV C_T values obtained from prawn pleopods from the infectivity trial to generate the material for gamma irradiation (A), and bioassays with untreated WSSV (B) and exposed to different doses of gamma irradiation (C to E).

1.2.2.3 Histological analysis of moribund prawns

Due to the high number of mortalities over a short period of time, and general lack of appearance of any gross signs of white spot disease prior to death, limited numbers of animals were collected for histology (n=34; Table 8). No pathology indicative of WSSV was observed in pre-screen or negative control prawns (Figure 9 to 13).

Table 8. Samples collected for histology and summary comments

Treatment of inoculum	Number of samples	Comments
Pre-screen	10	• Occasional focus of necrosis of cuticular epithelium, but no evidence of intranuclear inclusion bodies.
Negative controls	10	• No evidence of inclusion bodies (Figures 9, 10, 11)
WSSV Untreated	7	• 2dpi: Appear to be some developing inclusion bodies • 3dpi: Evidence of inclusion bodies in the cuticular epithelium of the foregut and exoskeleton, haematopoietic necrosis
Gamma irradiated to 10 kGy	7	• 2dpi: (2 prawns) Extensive necrosis of lymphoid organ lymphoid cells with karyorrhectic debris through tubule walls. Intranuclear inclusion bodies throughout foregut epithelium and at lower levels in the cuticular epithelium, associated with scattered foci of necrotic cells, haematopoietic tissue, and antennal gland epithelium. • 3dpi*: Numerous intranuclear inclusions in foregut and cuticular epithelium, associated with scattered foci of cell necrosis, and in the haematopoietic tissue. Occasional inclusions were present in antennal gland tubule epithelium. • 4dpi*: Intranuclear inclusions present in cuticular epithelium, with scattered foci of necrotic cells, and occasional inclusion in sub-epithelial connective tissue. Occasional inclusions were present in antennal gland tubule epithelium, also some scattered necrotic tubule epithelial cells. • 5dpi: Intranuclear inclusions scattered through the cuticular epithelium. Very occasional inclusions were present in antennal gland tubule epithelium.
Gamma irradiated to 15 kGy	4	• 1dpi: Extensive necrosis of lymphoid organ lymphoid cells and loss of clear tubule outline. Intranuclear inclusion bodies throughout foregut epithelium and scattered through foregut connective tissue and cuticular epithelium. Very occasional inclusion in antennal gland tubule epithelium. • 3dpi*: Intranuclear inclusions throughout the cuticular epithelium. Scattered inclusions present in thoracic connective tissues. Occasional inclusion in antennal gland tubule epithelium. • 6dpi*: Intranuclear inclusions scattered throughout the cuticular epithelium. The haematopoietic tissue contained many inclusions and occasional single necrotic cells. Occasional inclusions were present in antennal gland tubule epithelium, gill epithelium and interstitial connective tissue of the hepatopancreas.
Gamma irradiated to 20 kGy	6	• 3dpi: Widespread necrosis of the cuticular epithelium beneath the exoskeleton. Many necrotic cells have large intranuclear inclusion bodies consistent with WSSV infection. Inclusion bodies also in the antennal gland epithelium • 8dpi: Inclusion bodies in the cuticular epithelium and also in the haematopoietic tissue, and rarely in the antennal gland tubule epithelium.
Gamma irradiated to 25 kGy	10	• 3dpi: Numerous inclusion bodies in the cuticular and foregut epithelium throughout the prawn, and in haematopoietic tissue. Intranuclear inclusions present tin the foregut connective tissue. Occasional inclusions were present in antennal gland tubule epithelium.

		• 9dpi: Some inclusions and scattered necrotic cell nodules in foregut epithelium. Occasional inclusion bodies in cuticular epithelium, but many others in the sub-epithelial connective tissue, and in necrotic haematopoietic tissue. There is extensive necrosis of the lymphoid organ • 10dpi: Inclusion bodies in the cuticular epithelium of the foregut, and very occasionally in the epithelium of the cuticle. The foregut epithelium contained scattered nodule of necrotic cells. There is also extensive necrosis of the lymphoid organ (with occasional inclusion bodies), and numerous inclusion bodies in the haematopoietic tissue. • 15dpi – three prawns euthanised but live at the end of the trial: while most organs had no significant abnormalities, occasional intranuclear inclusions, probably due to WSSV infection, were detected in the antennal gland tubule epithelium in all three prawns.

* Limited organs present in histology slide for examination.

Inclusion bodies indicative of WSSV infection were observed in prawns exposed to WSSV-infected material, regardless of the dose of gamma irradiation with representative examples shown in Figures 12 to 16.

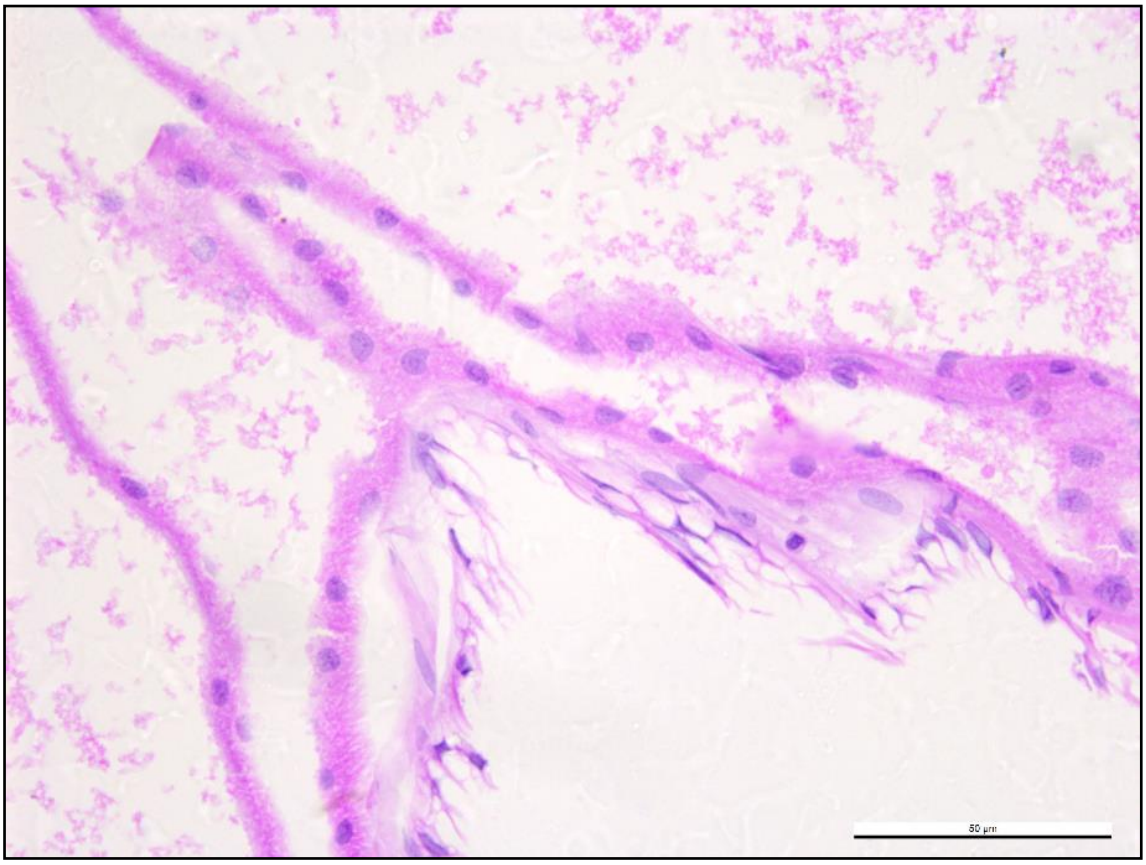


Figure 9. Negative control prawn - normal antennal gland

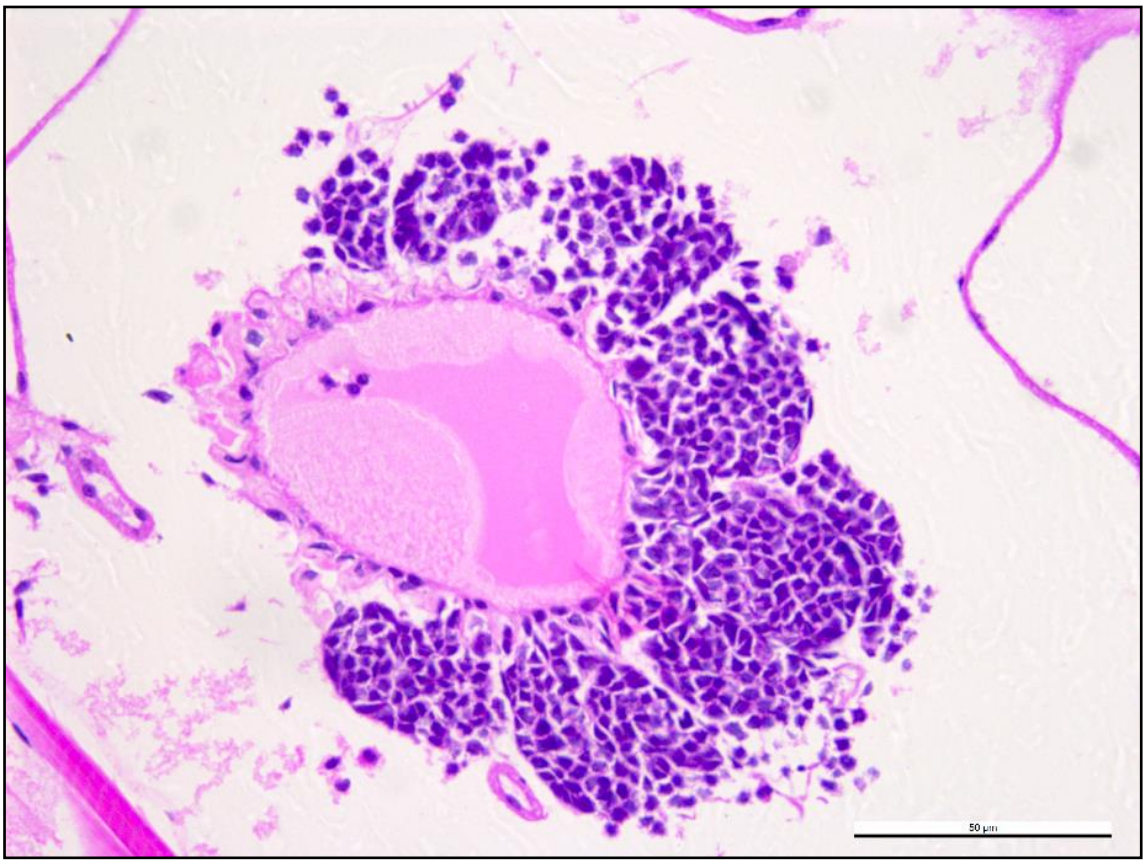


Figure 10. Negative control prawn - normal haematopoietic tissue

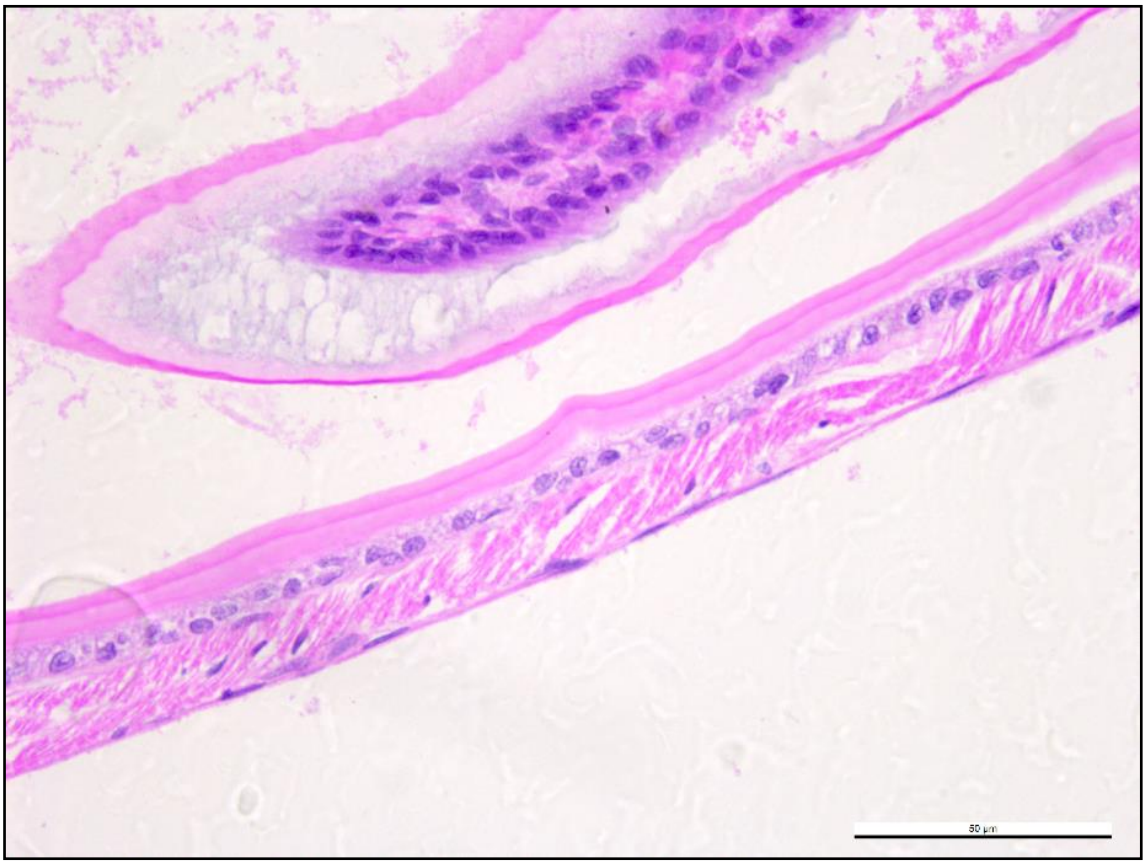


Figure 11. Negative control prawn - normal cuticular epithelium in the gut

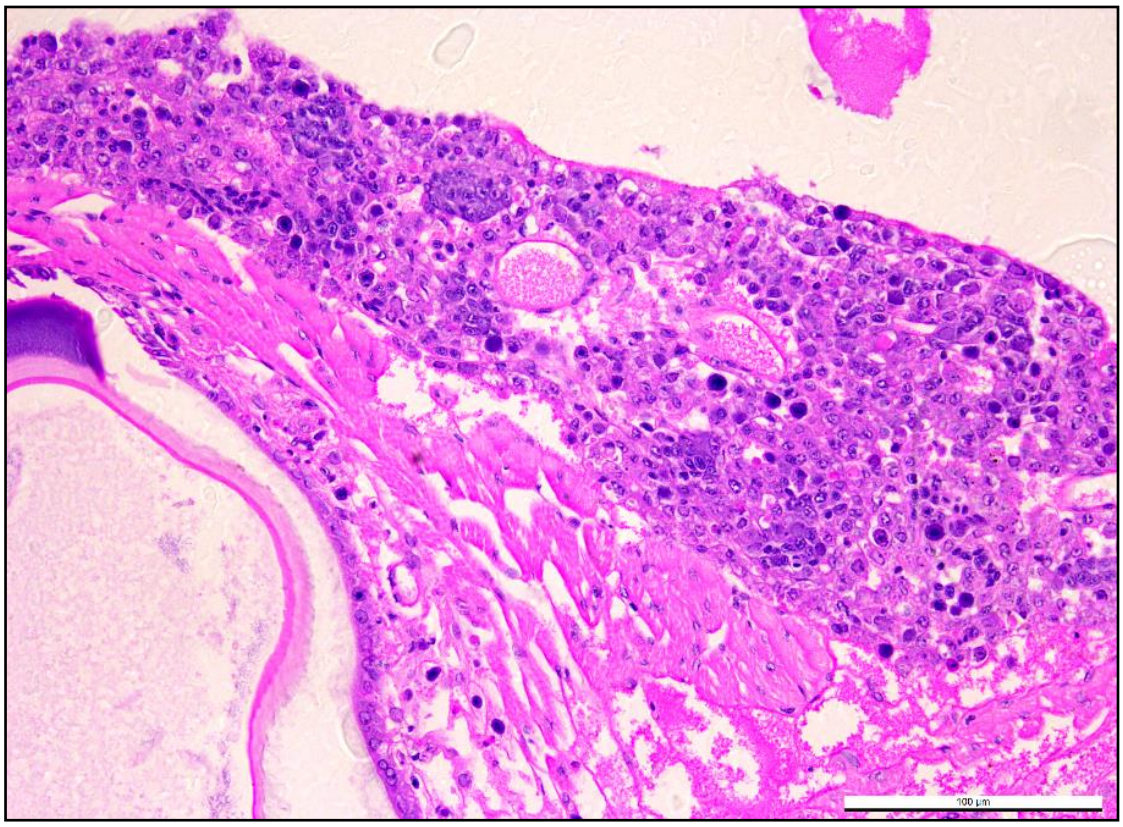


Figure 12. Prawn exposed to positive control WSSV-infected prawns - haematopoietic tissue

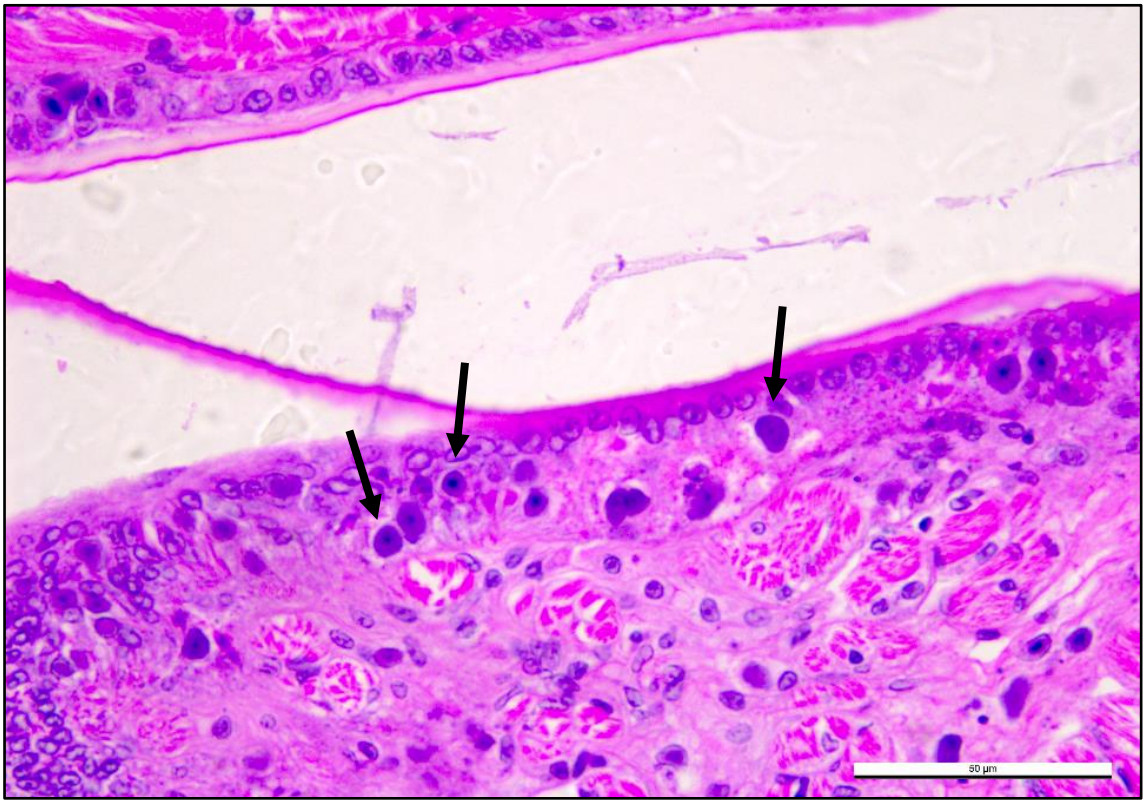


Figure 13. WSSV positive control prawn - inclusion bodies (arrows) in gut cuticular epithelium

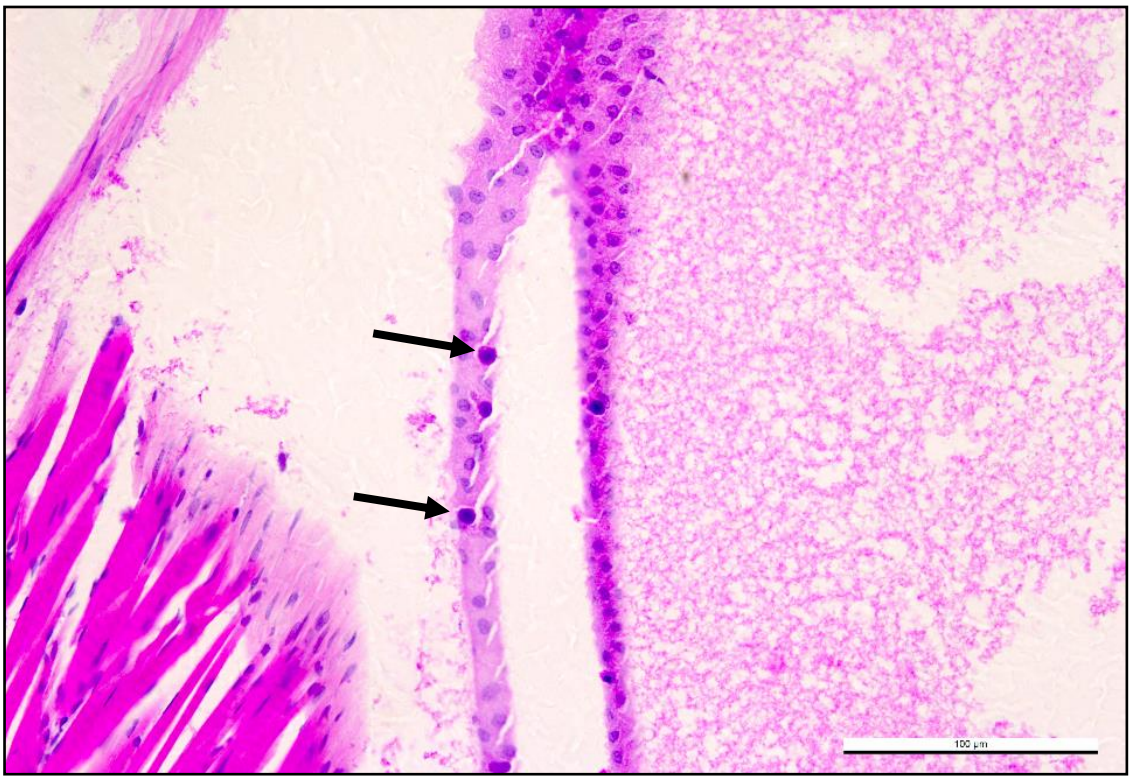


Figure 14. Prawn exposed to WSSV-infected prawns, gamma irradiated to 20 kGy – inclusion bodies (arrows) in antennal gland epithelium

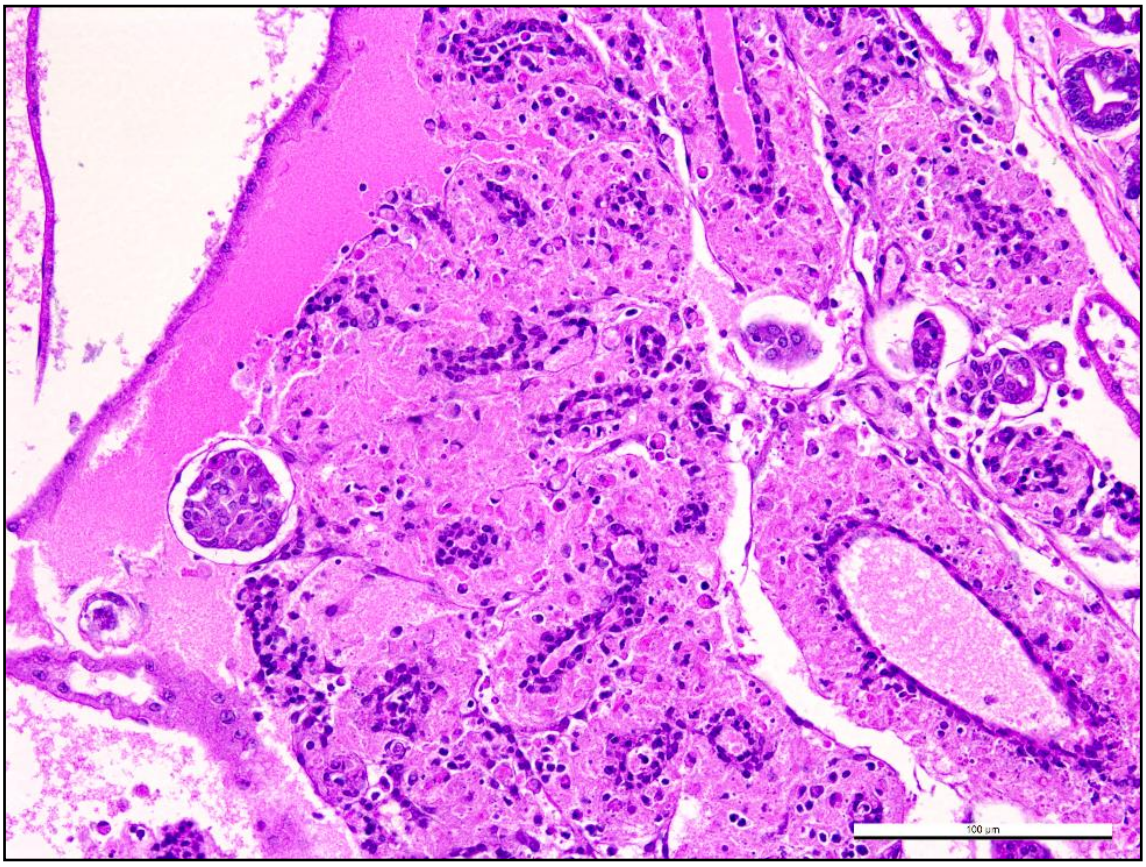


Figure 15. Prawn exposed to WSSV-infected prawns, gamma irradiated to 25 kGy - effacement of normal lymphoid organ architecture

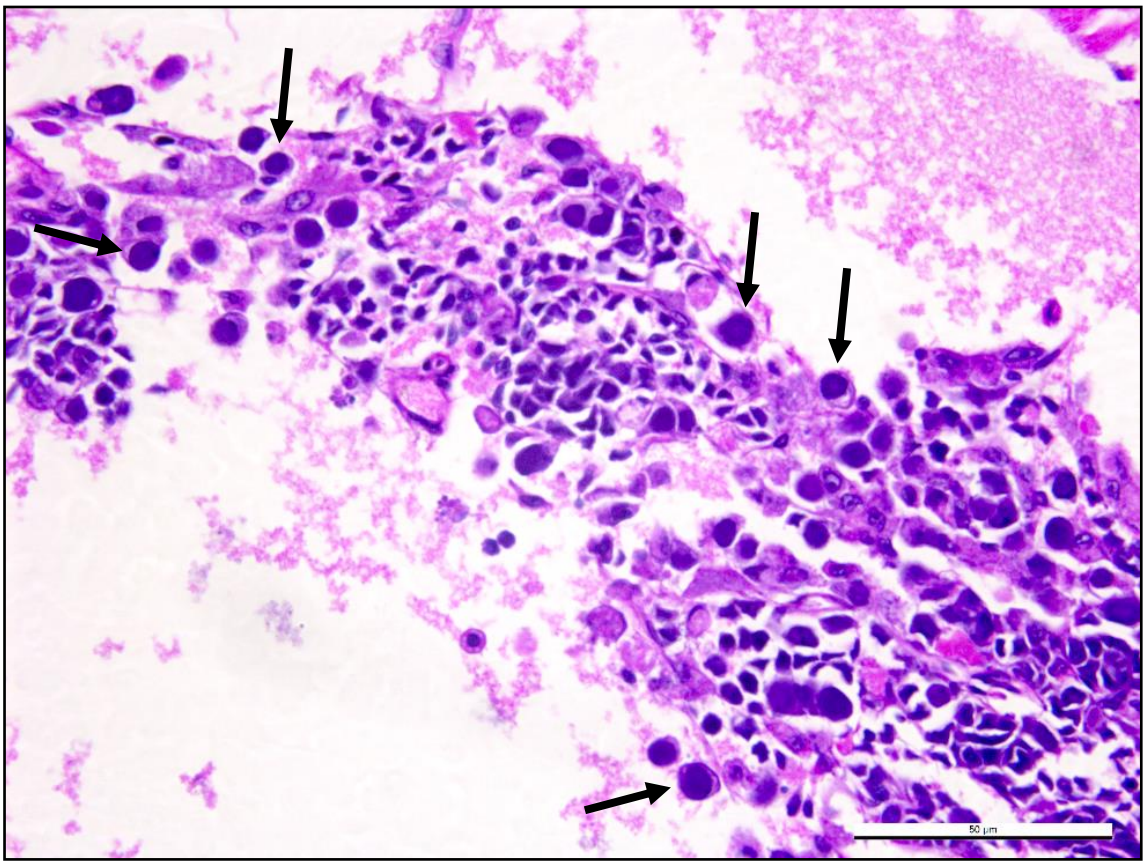


Figure 16. Prawn exposed to WSSV-infected prawns, gamma irradiated to 25 kGy - necrosis of haematopoietic tissue with inclusion bodies (arrows)

1.3. Conclusions

Based on mortality curves, molecular test results and histopathological analysis, gamma irradiation of prawns, clinically infected with WSSV, up to and including a dose of 25 kGy, failed to inactivate the infectious WSSV present.

Based on the results obtained during this trial, assessment of gamma irradiation at a dose of 50 kGy as a feasible method for treating prawns with White Spot Syndrome Virus was recommended and was approved as a variation to the project.

2. Experimental Trial 2: Assessment of gamma irradiation at 25 and 50 kGy

The aims of Experimental Trial 2 were:

1. To amplify WSSV in *P. monodon* and expose to gamma irradiation at 25 and 50 kGy.

2. To determine the effect of the two doses of gamma irradiation on the infectiousness of WSSV-infected *P. monodon* after exposure as follows:

    - Negative control prawns
    - 0 kGy: injected with WSSV-infected material and fed WSSV-infected material.
    - 25 kGy: fed WSSV-infected material after gamma irradiation to 25 kGy.
    - 50 kGy: injected and fed WSSV-infected material after gamma irradiation to 50 kGy.
    - 50 kGy: injected and fed WSSV-infected material after gamma irradiation to 50 kGy.
    - 50 kGy: fed WSSV-infected material after gamma irradiation to 50 kGy.

2.1. Generation of WSSV-infected *P. monodon* for gamma irradiation

2.1.1 Methods

2.1.1.1 Experimental prawns and commercial prawn feed

Prawns were sourced from a commercial farm. On arrival prawns were transferred to the secure ACDP LAF and placed in round polypropylene tanks containing approximately 80L seawater, a canister filter and air stones. Each tank held 25 to 30 prawns with water temperature maintained at 29 $\pm$ 1°C. Three replicate tanks were used in the rooms where prawns were exposed to WSSV. At least one tank in each room was fitted with a data-logger to measure temperature. Animals were monitored and fed daily and 30% water exchanges were undertaken every 2 days. After arrival, 10 negative control animals were sacrificed and pleopods fixed in 80% (v/v) EtOH for molecular testing and heads fixed in Davidson's fixative for histopathology. A bag of commercial prawn feed (Bag A) was also supplied and samples from the top (n=3), middle (n=3) and bottom (n=3) of the bag were taken for molecular testing for WSSV.

2.1.1.2 Preparation of inoculum and exposure of **P. monodon** *to WSSV*

Six replicate tanks were used to generate the WSSV-infected material for gamma irradiation with approximately 30 to 40 prawns (5 to 7 cm) in each tank.

For inoculation, prawns were caught with a net and held firmly to avoid tail-flick response. Each was inoculated with 50 µL of the inoculum into the second abdominal segment using a 26-gauge needle and placed back in the tank. The inoculum used was haemolymph collected from prawns infected with WSSV during Experimental Trial 1 which had been diluted 1/100 in PBS. Two tanks were set up for negative control prawns; prawns from one tank of negative controls were inoculated with 50 µL PBSA and no treatment was applied to the second negative control tank.

Prawns were monitored twice daily. For dead prawns, three pleopods were removed and placed into individually labelled 2 ml tubes containing 80% (v/v) EtOH. The rest of the prawn was pooled with a further 7 to 10 dead prawns in a labelled zip-lock bag in a plastic container and stored at -80°C. At the end of the trial a small number of surviving prawns were bled from the ventral sinus using a 26-gauge needle into a syringe containing 10% sodium citrate. For moribund prawns, three pleopods were removed and placed into

individually labelled 2 ml O-ring, screw-cap tubes containing 80% (v/v) EtOH. The head was then injected with Davidson's Fixative and placed in a labelled 50 ml specimen container containing fixative. After 24 hours tissues were transferred to 70% (v/v) EtOH. Tissues for histology were processed for paraffin-embedding, sectioning and H&E staining using standard procedures. Sections were examined using a compound light microscope or digitally scanned using a whole slide imaging system and viewed on a computer

2.1.1.3 Molecular testing for WSSV

2.1.1.3.1 Prawn tissues

A sub-sample of one pleopod from each prawn was added to a bead beating tube containing 600 µl PBS and homogenised using a FastPrep-24 bead-beater (MP Biomedicals). Homogenates were clarified by centrifugation at 10 000 × g for 5 min. Total nucleic acid was extracted from 200 µl of the homogenate supernatant using the MagMAX™ CORE Nucleic Acid Purification Kit on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Life Technologies). Nucleic acid was eluted in 90 µl elution buffer and stored at -80°C prior to testing. For nucleic acid extraction from haemolymph, 50µL was extracted using the QIAamp Viral RNA Mini Kit and eluted in 60µL. A negative extraction control was included with each extraction run for all sample types. The CSIRO WSSV qPCR assay was performed as described in Section 1.1.1.4. The CSIRO Shrimp EF1 qPCR, which uses the primers and probe described by Cowley et al., (2018) and the same reaction and cycling conditions as the CSIEO WSSV qPCR, was used as a housekeeping gene assay for prawn samples, with samples tested in singlicate. The negative extraction control, no template control and positive controls were included on each qPCR plate with results analysed using a cycle threshold of 0.1. Any sample generating a typical amplification curve was deemed positive and each PCR plate run was deemed to be valid if negative and positive control results were as expected

2.1.1.3.2 Commercial prawn feed

Following the 2016 WSSV disease outbreak in SE Queensland, AFDL has undertaken testing for WSSV of samples other than tissues from penaeid prawns (e.g. prawn feed and prawn feed ingredients). Using a range of internal PCR controls, and multiple pathogen-specific tests, many of these atypical samples did, indeed, contain WSSV nucleic acid. For this reason, samples from two bags (Bag A and Bag B) of commercial prawn feed provided with the prawns used in the bioassays was screened for WSSV. Initially, a single sample of feed was tested from Bag A with additional samples subsequently taken from the top, middle and bottom of Bag A and Bag B.

Feed samples (30mg) were homogenised by bead beating as described above (Section 1.1.3.1) with 140µL of homogenate from each sample added to 560µL AVL buffer containing 5µL of stock T4 DNA phage. Samples were processed using the QIAamp Viral RNA Mini Kit and nucleic acids eluted in 60µL. Samples were screened using the CSIRO WSSV qPCR, OIE WSSV qPCR and Shrimp EF1 qPCR assays as described above (Section 1.1.3.1) with the artificial probe also added to the WSSV-specific qPCR reactions at a concentration of 250 nM. The artificial probe as added as an additional risk-mitigation measure to ensure samples testing positive for WSSV were not due to contamination with the positive control plasmids, specific for each assay (Moody et al., 2010). Samples were tested in duplicate for both WSSV qPCR assays and in singlicate for the Shrimp EF1 and T4 assays. Conventional PCR testing, using an AFDL in-house assay (Durand-East WSSV nPCR) was undertaken of samples testing positive by the WSSV-specific qPCR assays. The Durand-East WSSV nPCR uses the primer sequences from the OIE WSSV qPCR (Durand and Lightner, 2002) and CSIRO WSSV qPCR (East et al., 2006), with some modifications (Table 9) to generate primary and nested PCR amplicons of 427bp and 323bp, respectively. Amplicons were visualised after electrophoresis on 1.5% agarose gels stained with SYBR Safe DNA Gel Stain (Life Technologies). Amplicons of the expected size were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Each amplicon was sequenced using forward and reverse primers by direct product sequencing using the BigDye Terminator v3.1 Cycle Sequencing chemistry and

3130xl Genetic Analyzer. Sequence analysis and comparison with WSSV reference sequences was undertaken using Geneious Pro (Biomatters).

Table 9. Primer sequence used in the Durand-East WSSV nPCR

First step Primers	Sequence
WSSV-F	5'- CCG ACG CCA AGG GAA CT -3'
WSSV OIE 1079R	5'- GCT GCC TTG CCG GAA ATT A -3'

Nested Primers	Sequence
DE-WSSVn-Rrc	5'- TGG AAA CGG TAA CGA ATC TGA A -3'
DE-WSSVn-1011Frc	5'- CTG AGA TGA GGA CGG GAC CA -3'

rc = reverse compliment

2.1.1.4 Gamma irradiation of WSSV-infected P. monodon

WSSV-infected prawns were removed from the secure area of ACDP and stored at -80°C. Prior to transport, five replicate 80L Techniice eskies were prepared. Each esky contained four pots of WSSV-infected prawns and dry ice. Four eskies were transported to Steritech for gamma irradiation, one irradiated to 25 kGy and three irradiated to 50 kGy. The fifth esky was retained at ACDP as a WSSV positive infection control. Each esky was labelled with the target dose.

After receipt of the eskies containing the infected samples at Steritech, a dosimeter was placed on each esky in the reference position. Each esky was then topped-up with dry ice to bring its total weight up to 65kg and then irradiated in Steritech's IR-217 Cobalt-60 gamma irradiator. Each esky was given a radiation exposure time estimated to deliver a maximum absorbed dose to the samples as close to but not exceeding the target dose. Following irradiation, the reference dosimeter was removed, its absorbed dose measured, and the absorbed doses delivered to the samples calculated.

The irradiated eskies were then transported back to ACDP. Upon return to ACDP, the prawns were removed from the eskies and returned to -80°C storage in the secure area.

2.1.2 Results and Discussion

2.1.2.1 Exposure of P. monodon to WSSV

One day post-exposure, across all six replicate tanks, one prawn was found dead (2.9% mortality). Two days post-exposure 37 prawns were found dead (17.2% cumulative mortality), three days post-exposure 115 prawns were found dead (71.2% cumulative mortality) and four days post exposure 50 prawns were found dead (94.4% cumulative mortality). Haemolymph was bled from the surviving 12 prawns and the experiment was terminated. Mortality curves for each replicate tank were very consistent and are depicted in Figure 17. There were 215 WSSV-infected prawns stored for gamma irradiation. There were no mortalities in negative control tanks.

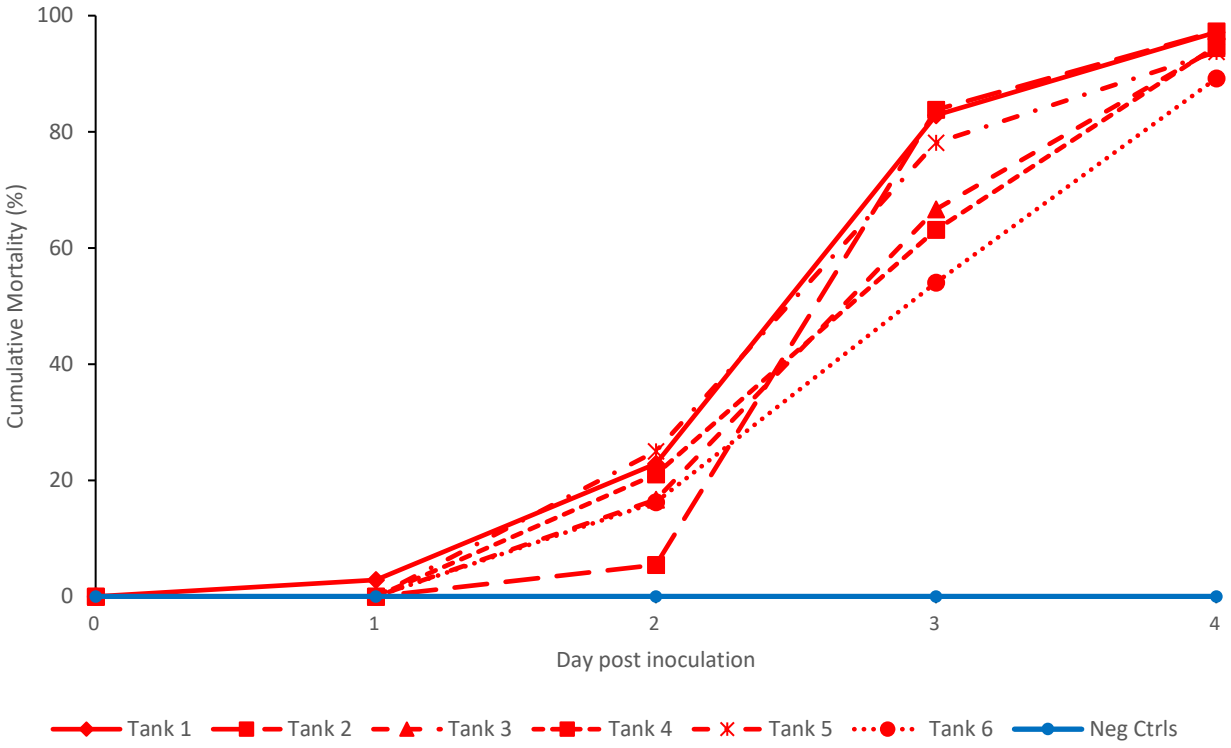


Figure 17. Cumulative mortality after exposure to WSSV by inoculation, to generate material for gamma irradiation.

2.1.2.2 Molecular testing for WSSV

2.1.2.2.1 Prawns

All the prawns injected with WSSV were CSIRO WSSV qPCR test positive with mean CSIRO WSSV qPCR C_T values across the six replicate tanks ranging from 12.17 to 15.19 (Table 10). Two prawns from the untreated negative control tank tested Indeterminate for WSSV with duplicate qPCR results of 36.55/negative and 35.36/negative, respectively. This was unexpected given the protocols in place for *in vivo* work with aquatic animals in the ACDP LAF, where negative control animals are observed and maintained first, are held in separate rooms from animals exposed to pathogenic agents, and a three minute shower is required on exit from rooms where animals are exposed to pathogenic agents

Table 10. CSIRO WSSV qPCR results for *P. monodon* used to generate WSSV-infected material

Treatment	Number positive/ Number tested	CSIRO WSSV qPCR Mean C_T	CSIRO WSSV qPCR C_T range
Pre-trial	0/10	Negative	Not applicable
Inoculum	1/1	15.19	Not applicable
Injected - Tank 1	35/35	13.12	10.16 – 18.76
Injected - Tank 2	37/37	13.46	11.12 – 17.78
Injected - Tank 3	34/34	12.71	9.65 – 17.09
Injected - Tank 4	36/36	13.14	11.02 – 16.57
Injected - Tank 5	30/30	13.70	11.35 – 20.91
Injected - Tank 6	37/37	12.17	9.57 – 16.14
Negative - PBSA inject	0/25	Negative	Not applicable
Negative – no treatment	2 Indeterminate/25	36.55, 35.36	35.36 – 36.55

2.1.2.2.2 Commercial prawn feed

Initial feed samples taken from Bag A tested positive for WSSV using the CSIRO WSSV qPCR, with mean CT value of 33.5 and 32.9. Subsequent samples taken from the top, middle and bottom of Bag A were also test positive by both the CSIRO and OIE WSSV qPCR assays (Table 11). The narrow range of C_T values indicated that WSSV template is homogenously distributed throughout each bag. Samples from Bag A generated amplicons of the expected size in the nested PCR of the AFDL in-house assay (Durand-East WSSV nPCR) which shared 100% nucleotide identity with WSSV reference sequences.

Table 11. Molecular test results after testing samples from feed Bag A

Sample	CSIRO WSSV qPCR	OIE WSSV qPCR	Artificial Probe qPCR	T4 Phage qPCR	Shrimp EF1 qPCR
Bag A: Top 01	33.64	34.64	Negative	27.74	33.58
Bag A: Top 02	32.63	34.20	Negative	27.56	32.64
Bag A: Top 03	32.79	34.07	Negative	27.87	33.25
Bag A: Middle 01	32.70	33.88	Negative	28.11	31.59
Bag A: Middle 02	32.65	33.38	Negative	28.43	31.92
Bag A: Middle 03	32.65	33.88	Negative	29.13	31.67
Bag A: Bottom 01	33.40	34.34	Negative	28.49	32.38
Bag A: Bottom 02	33.33	34.22	Negative	29.36	32.93
Bag A: Bottom 03	33.71	34.94	Negative	28.45	33.09
Mean	**33.06**	**34.17**		**28.35**	**32.56**
Range	**32.63 – 33.71**	**33.38 – 34.94**		**27.56 – 29.36**	**31.59 – 33.58**
NEC	Negative	Negative	Negative	27.53	Negative
NTC	Negative	Negative	Negative	Negative	Negative
POS Ctrl 1	23.63	Not tested	24.16	Negative	Negative
POS Ctrl 2	30.50	Not tested	31.04	Negative	Negative
POS Ctrl 3	Not tested	27.15	26.03	Negative	Negative
POS Ctrl 4	Not tested	33.94	32.84	Negative	Negative

NEC = Negative extraction control; NTC = No template control

2.1.2.3 Histology of WSSV-infected P. monodon

In all cases, only the cephalothorax of the prawn was examined via mid-sagittal and/or oblique sections. A minimum of two H&E-stained sections were examined per animal.

No inclusion bodies typical of WSSV were observed in any pre-trial samples. The spheroids observed within the lymphoid organ of 6 of the prawns are non-specific but may indicate background infection, possibly with *gill associated virus* (GAV ≡ YHV2). All prawns had adequate lipid vacuoles throughout the hepatopancreas.

The negative control groups had no evidence of WSSV infection.

The prawns that had been exposed to WSSV via intra-muscular inoculation showed histopathological lesions consistent with WSSV infection, including necrosis of epithelial cells of the integument, gastric epithelium, and lymphoid organ, along with characteristic basophilic intranuclear inclusion bodies. Whilst autolysis obscured the ability to interpret the histopathology in some individual prawns, pathology consistent with WSSV infection was present in each infected tank. Specific histological findings are described in Table 12.

Table 12. Specific histological findings in prawns

Pre-trial samples

Prawn ID	Histopathology	WSSV inclusions
1	No significant findings	None
2	Focal, mild haemocyte infiltrates within skeletal muscle	None
3	Lymphoid organ: multiple spheroids of different stages comprising 50 % of the parenchyma with necrotic debris	None
4	Lymphoid organ: Spheroids comprise 50% of the parenchyma	None
5	Lymphoid organ: Spheroids comprise 30% of the parenchyma (Figure 18)	None
6	No significant findings	None
7	Lymphoid organ: Small numbers of spheroids comprise 10% of the parenchyma	None
8	Lymphoid organ: There is a single early spheroid	None
9	No significant findings	None
10	Lymphoid organ: Spheroids comprise 10% of the parenchyma	None

Negative controls: No treatment

Prawn ID	Histopathology	WSSV inclusions
1	No significant findings	None
2	No significant findings	None

Negative controls: Injected with PBS

Prawn ID	Histopathology	WSSV inclusions
1	No significant findings	None
2	Lymphoid organ: Spheroids comprise 50% of the parenchyma	None

Room C7 Tank 1: Injected with WSSV

Prawn ID	Histopathology	WSSV inclusions
6	There is moderate autolysis with intranuclear, Cowdry type A inclusions in subcuticular gastric epithelium	Positive
9	There are multiple bacterial colonies throughout a number of tissues with marked lymphoid organ autolysis despite only mild autolysis of the hepatopancreas. INIBs are widespread in multiple tissues.	Positive

Room C7 Tank 2: Injected with WSSV

Prawn ID	Histopathology	WSSV inclusions
2	No significant abnormalities were observed, however there was diffuse autolysis throughout.	Too autolysed to comment
4	Lymphoid organ: diffuse, mild necrosis with INIBs in multiple tissues (Figure 19)	Positive

Room C7 Tank 3: Injected with WSSV

Prawn ID	Histopathology	WSSV inclusions
3	Lymphoid organ: marked autolysis with spheroid formation. Small numbers of INIBs in multiple tissues.	Positive
17	Haematopoietic tissue: there is mild disruption to the normal architecture of multiple clusters of haematopoietic tissue, along with intranuclear inclusion bodies Lymphoid organ: the normal architecture of the lymphoid organ is disrupted by expansion of the interstitial sinuses, dilation of tubular lumens and moderate necrotic debris. INIBs are present in multiple tissues.	Positive

Room C7 Tank 4: Injected with WSSV

Prawn ID	Histopathology	WSSV inclusions
1	Tissues are too autolysed for comment	Too autolysed for comment
2	No significant findings. No lymphoid organ is present. INIBs in multiple tissues.	Positive

Room C7 Tank 5: Injected with WSSV

Prawn ID	Histopathology	WSSV inclusions
1	Multifocal necrosis of subcuticular gastric epithelium, connective tissue and epidermis. No lymphoid organ is present. INIBs are widespread in multiple tissues.	Positive
2	There is diffuse necrosis of the lymphoid organ with multifocal necrosis of the gastric subcuticular epithelium. INIBs are widespread in multiple tissues.	Positive

Room C7 Tank 6: Injected with WSSV

Prawn ID	Histopathology	WSSV inclusions
1	INIBs are present in multiple tissues. There is moderate autolysis and no lymphoid organ is present.	Positive
2	Tissues are too autolysed for comment.	Too autolysed for comment.

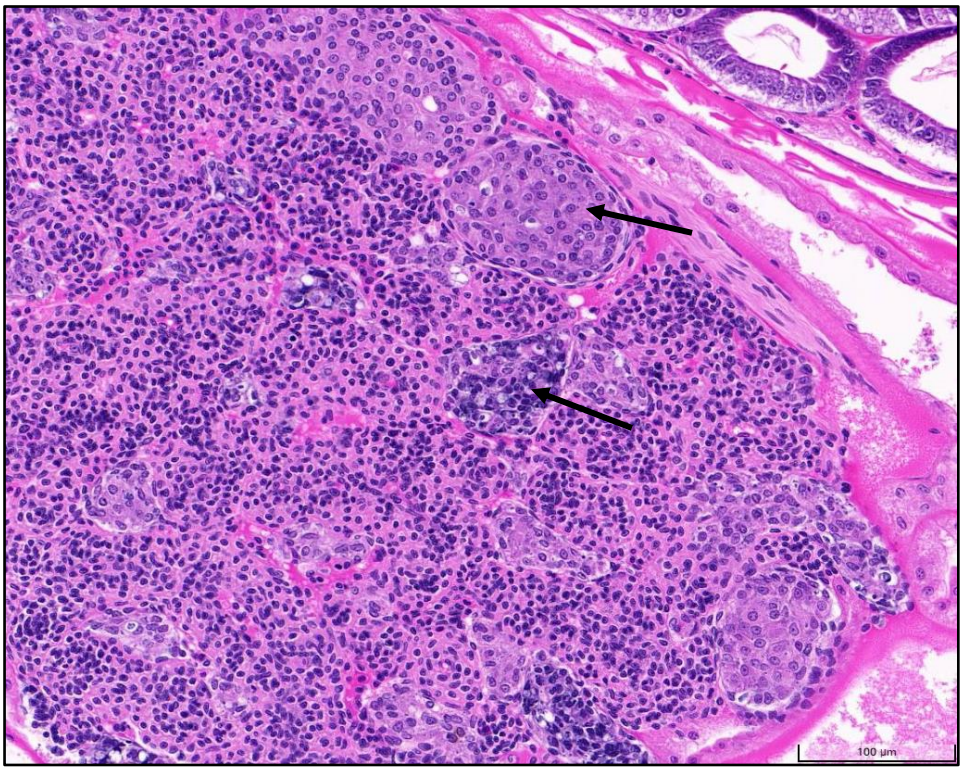


Figure 18. Lymphoid organ with spheroid formation (arrows) in a pre-trial prawn.

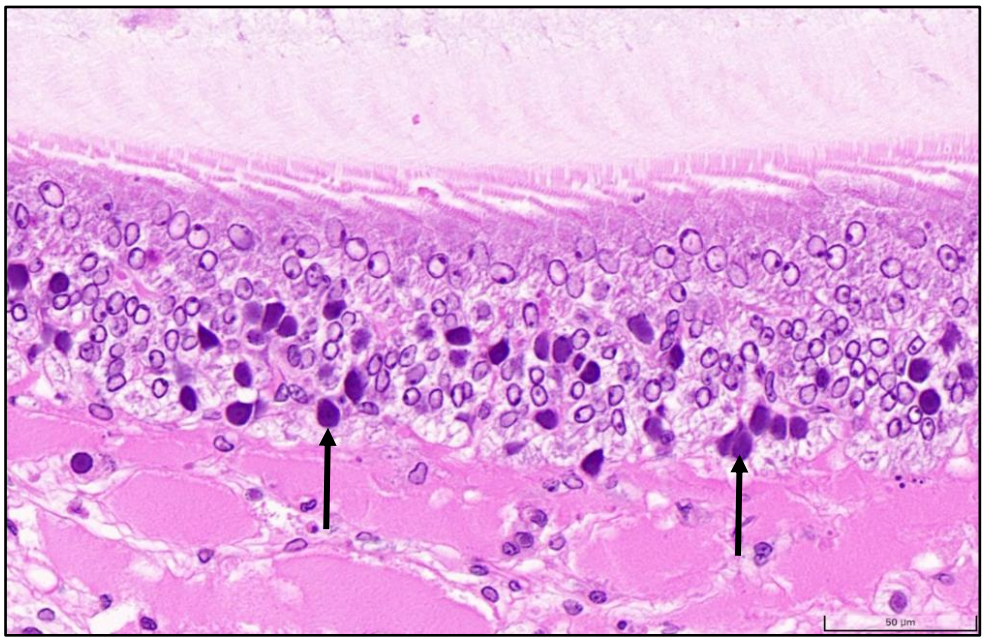


Figure 19. Numerous intranuclear inclusion bodies (arrows) within the gastric epithelium in a prawn injected with WSSV

2.1.2.4 Gamma irradiation of WSSV-infected P. monodon

WSSV-infected prawns were removed from the secure area of ACDP and stored at -80°C. Prior to transport, five replicate 80L Techniice eskies were prepared. Each esky contained four pots of WSSV-infected prawns and dry ice. Four eskies were transported to Steritech for gamma irradiation at 25, 50, 50 and 50 kGy with the fifth esky retained at ACDP as a positive infection control. Each esky was labelled with the target dose.

After receipt of the eskies containing the infected samples at Steritech, a dosimeter was placed on each esky in the reference position. Each esky was then topped-up with dry ice to bring its total weight up to 65kg and then irradiated in Steritech's IR-217 Cobalt-60 gamma irradiator. Each esky was given a radiation exposure time estimated to deliver a maximum absorbed dose to the samples as close to but not exceeding the target dose. Following irradiation, the reference dosimeter was removed, its absorbed dose measured, and the absorbed doses delivered to the samples calculated.

The irradiated eskies were then transported back to ACDP. Upon return to ACDP, the prawns were removed from the eskies and returned to -80°C storage in the secure area.

2.2. Bioassay of WSSV-infected material after gamma irradiation at 25 and 50 kGy

2.2.1 Methods

2.2.1.1 Experimental prawns

Prawns were sourced from a commercial farm. On arrival prawns were transferred to the secure ACDP LAF and placed in round polypropylene tanks containing approximately 80L seawater, a canister filter and air stones. Each tank held 25 to 30 prawns with water temperature maintained at 29 ± 1°C. Three replicate tanks were used in the rooms where prawns were exposed to WSSV. At least one tank in each room was

fitted with a data-logger to measure temperature. After arrival, 10 negative control animals were sacrificed and fixed in 80% (v/v) EtOH for molecular testing as described above and in Davidson's fixative for histopathology. Animals were monitored and fed daily with s second bag of commercial prawn feed (Bag B) supplied with the prawns. Ten samples were taken from the top (n=3), middle (n=3) and bottom (n=4) of the bag were taken for molecular testing for WSSV. Thirty precent water exchanges were undertaken every 1 to 2 days.

2.2.1.2 Preparation of inoculum

Inoculum from the untreated WSSV-infected prawns and gamma irradiated material was prepared as described above (Section 2.1.2)

2.2.1.3 Inoculation and feeding of prawns and sampling

Prior to inoculation, 10 negative control animals were sacrificed. Pleopods were fixed in 80% (v/v) EtOH for molecular testing and the head was fixed in Davidson's Fixative for histological analysis. For inoculation, prawns were caught with a net and held firmly to avoid tail-flick response. Initially, each prawn was inoculated with 50 µl of the inoculum into the second abdominal segment using a 26-gauge needle and placed back in the tank.

Depending on the treatment (Table 13), from Day 2 the remaining gamma irradiated prawns (from the same gamma irradiation dose as the inoculum) were fed to the prawns in each of the three replicate tanks that had been inoculated on Day 1. For each tank, 3 frozen prawns in a zip-lock bag were cut in half longitudinally with a sterile scalpel, then sliced into small pieces and fed to the prawns in the tanks. The feeding of the irradiated prawns was undertaken to ensure that prawns were exposed to all the gamma irradiated material (in the event that no mortalities were observed). Feeding of prawn tissue continued until Day 6 or 7 post infection when all the frozen WSSV-infected, gamma irradiated prawn tissue has been consumed. After this time point, all prawns were fed commercial prawn feed. Negative control prawns were fed commercial prawn feed from Day 1.

Table 13. Different treatment regimens used to expose *P. monodon* to gamma irradiated WSSV.

Treatment	Room	Replicate tanks	Number of prawns
Pre-screen	Euthanased in arrival	Not applicable	20
Negative controls	LAF Room C8	2	2 x 30
WSSV positive controls	LAF Room C7	3	3 x 30
25 kGy: Feed only	LAF Room C6	3	3 x 30
50 kGy: Inject and feed	LAF Room C5	3	3 x 30
50 kGy: Inject and feed	LAF Room C3	3	3 x 30
50 kGy: Feed only	LAF Room C3	3	3 x 30
		Total	520

Prawns were observed daily. When prawns were found dead, two pleopods were removed and placed into individually labelled 2 ml O-ring, screw-cap tubes containing 80% (v/v) EtOH. The rest of the prawn was pooled with other dead prawns in a labelled zip-lock bag in a plastic container and stored at -80°C. For observed moribund prawns, two pleopods were removed and placed into individually labelled 2 ml O-ring, screw-cap tubes containing 80% (v/v) EtOH. The head was then cut in half longitudinally with both halves put in Davidson's Fixative in a labelled 50 ml specimen container. Alternatively, the head was injected with Davidson's Fixative and placed in a labelled 50 ml specimen container containing fixative. After 24 hours tissues were transferred to 70% (v/v/) EtOH. Tissues for histology were processed for paraffin-embedding, sectioning and H&E staining using standard procedures. Sections were examined using a compound light microscope or digitally scanned using a whole slide imaging system and viewed on a computer.

2.2.1.4 Processing and molecular testing for WSSV

Samples were screened using the CSIRO WSSV qPCR and Shrimp EF1 qPCR assays as described above (Section 1.1.3.1) with the artificial probe also added to the WSSV-specific qPCR reactions at a concentration of 250 nM. The artificial probe was added as an additional risk-mitigation measure to ensure samples testing positive for WSSV were not due to contamination with the positive control plasmids (Moody et al., 2010). Samples were tested in duplicate for the CSIRO WSSV qPCR assays and in singlicate for the Shrimp EF1 and T4 assays.

2.2.2 Results and Discussion

2.2.2.1 Preparation of inoculum and exposure of P. monodon *to gamma irradiated WSSV-infected prawns*

Result of testing the nucleic acid extracted from the inoculums using the CSIRO assays is presented in Table 14. Aliquots were taken immediately after preparation (pre-inoculation) and after prawns had been injected in the ACDP LAF (post-inoculation). The narrow C_T range (14.13 to 14.54, post-inoculation) demonstrates the uniformity of the different inoculum preparations but is not a measure of the level of infectivity.

Table 14. CSIRO WSSV qPCR results for each inoculum used.

Inoculum	CSIRO WSSV qPCR Mean C_T Pre-inoculation	CSIRO WSSV qPCR Mean C_T Post-inoculation
WSSV - untreated	13.83	14.16
WSSV - 50 kGy inject and fed	13.95	14.54
WSSV - 50 kGy inject and fed	13.88	14.13

In prawns exposed to the WSSV positive control material (i.e. no gamma irradiation treatment), mortalities commenced Day 2 post-exposure and reached 100% mortality on Day 4. At the termination of the trial on Day 29 post-exposure, mortalities in negative control prawns and all prawns exposed to gamma irradiated WSSV-infected prawns, by either feeding or injection and feeding, was <4% (Table 15, Figure 20).

Table 15. Mean cumulative mortality in prawns exposed to untreated and gamma irradiated WSSV.

Inoculum	Number dead/number exposed	Cumulative mortality
Negative controls	1/52	1.92%
WSSV – positive controls	92/92	100%
WSSV - 25 kGy fed	1/77	1.30%
WSSV - 50 kGy inject and fed	3/83	3.62%
WSSV - 50 kGy inject and fed	2/82	2.44%
WSSV - 50 kGy fed	3/76	3.95%

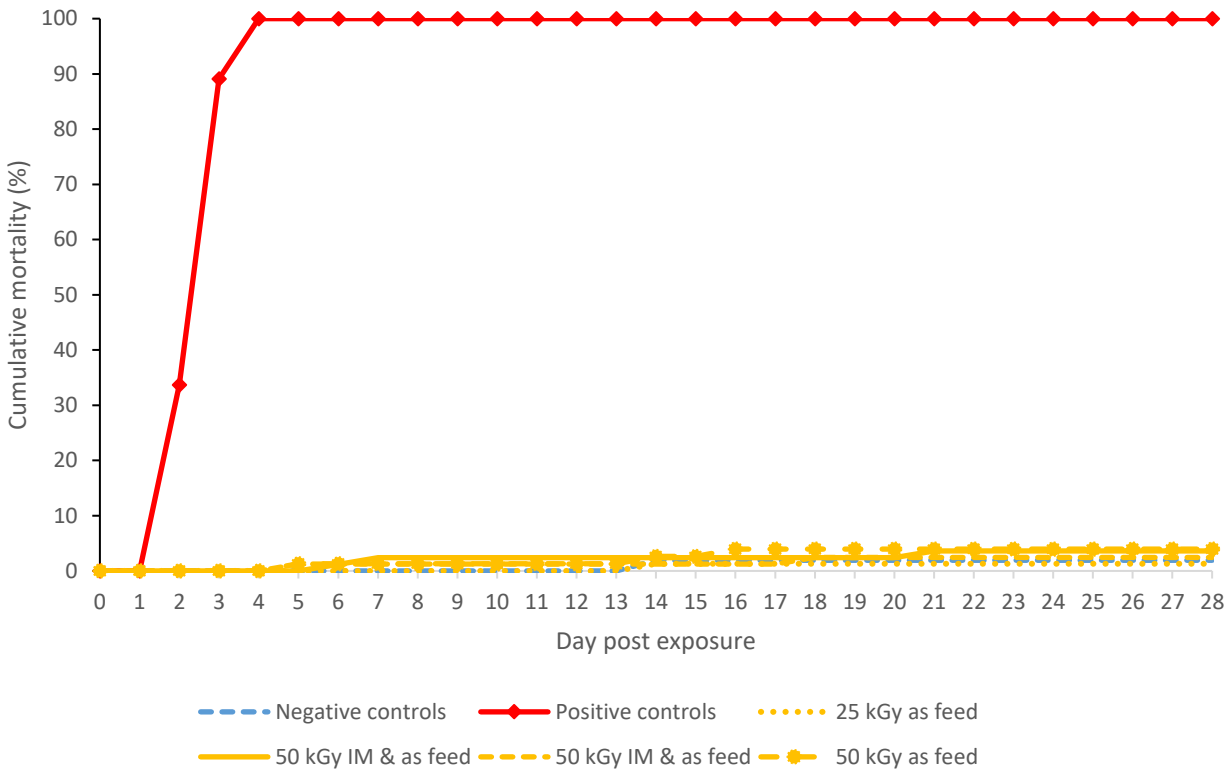


Figure 20. Mean cumulative mortality in prawns exposed to untreated and gamma irradiated WSSV.

2.2.2.2 Molecular testing for WSSV

Unexpected CSIRO WSSV qPCR results were generated, with the exception of *P. monodon* exposed to prawns infected with WSSV; as 100% mortalities had occurred by 4 days post exposure, mean C_T values across the three replicate tanks were 12.63, 12.28 and 12.57, respectively (Table 17). Positive CSIRO WSSV qPCR results were obtained from *P. monodon* sampled at the termination of the trial in Day 28 from prawns exposed to gamma irradiated prawns at both 25kgy and 50 kGy, after injection and feeding and after exposure to prawns by feeding only. In three of four treatments, only 3 (4%), 10 (12%) and 9 (16%) prawns, respectively, were CSIRO WSSV qPCR test positive. In contrast, in one treatment 64 (78%) prawns were CSIRO WSSV qPCR test positive. Interestingly, only one negative control prawn tested Indeterminate. Regardless, all CSIRO WSSV qPCR mean CT values, for all gamma irradiated WSSV prawn exposure treatments, were >34.02 (Table 16). All artificial probe (AP qPCR) results were test negative, indicating the source of the unexpected CSIRO WSSV qPCR positive test results was not due to laboratory contamination during WSSV qPCR testing.

2.2.2.1 Molecular testing of commercial prawn feed for WSSV

All ten samples taken from the top, middle and bottom of Bag B tested positive for WSSV using the CSIRO WSSV qPCR, with mean C_T values of 30.35 to 31.57 (Table 17). The narrow range of C_T values indicated that WSSV template is homogenously distributed throughout each bag. Samples from Bag B generated amplicons of the expected size in the nested PCR of the AFDL in-house assay (Durand-East WSSV nPCR) which shared 100% nucleotide identity with WSSV reference sequences.

Given that the unexpected CSIRO WSSV qPCR C_T values in these prawns were higher than those detected in the feed (Figure 21) it is possible that the source of the positive WSSV test results were due to contamination of WSSV DNA from the commercial prawn feed or residual gamma irradiated WSSV

inoculum/feed introduced at the beginning of the trial. The possibility of the positive CSIRO WSSV qPCR test results, due to other potential sources of infectious WSSV or WSSV template, were considered highly unlikely and included:

1. *Staff entering the ACDP LAF Row C where the* in vivo *work was undertaken*. Prior to entry to the LAF Row C, all clothing is removed and staff don either scrubs, overalls, or a dressing gown prior to entering the LAF Row C clean corridor. The room containing the negative control animals is always entered before any room where infectious material may have been used. If either scrubs or overalls are worn into a room where infectious material has been used, this clothing is left in the room before exiting.

2. *Staff moving from one room ACDP LAF Row C to another*. Prior to exiting a room where infectious material has been used, all clothing is removed, and staff have a 3-minute shower. This 3-minute shower is identical to that required on exiting the ACDP secure area. If staff are to enter another room, a dressing gown is worn and removed prior to entry of the next room, or scrubs or overalls are donned and left in the next room, prior to exit (as described above).

3. *Staff processing and homogensing samples prior to nucleic acid extraction.* Samples are processed in an Isolation Room which has an air-lock entry. On completion of work, the Class II Biological Safety Cabinet used to process the samples is decontaminated according to ACDP PCR 3 Standard Operating Procedures. Only samples from one treatment room are processed at a time.

4. Cross-contamination during nucleic acid extraction. Samples are processed in a Class II Biological Safety Cabinet which is decontaminated according to ACDP PCR 3 Standard Operating Procedures. Only samples from one treatment room are extracted at a time, preventing cross-contamination between the different treatment groups.

Table 16. Molecular test results for all samples generated during the trial

Treatment	CSIRO WSSV qPCR					AP qPCR	Shrimp EF1 qPCR C_T
	Number tested	Number Positive	Number Indeterminate	Number Negative	Mean C_T (range)		
Pre-screen	10	0 (0%)	0 (0%)	10 (100%)	Not applicable	Negative	20.52
Negative controls (Tank 1)	26	0 (0%)	1 (3.9%)	25 (96.2%)	36.58	Negative	20.49
Negative controls (Tank 2)	26	0 (0%)	0 (0%)	26 (100%)	Not applicable	Negative	19.93
Total	**62**	**0**	**1**	**61**	**36.58**	**Negative**	**20.31**
WSSV positive controls (Tank 1)	30	30 (100%)	0 (0%)	0 (0%)	12.63 (10.10-15.69)	Negative	24.33
WSSV positive controls (Tank 2)	32	32 (100%)	0 (0%)	0 (0%)	12.28 (10.43-15.70)	Negative	23.01
WSSV positive controls (Tank 3)	30	30 (100%)	0 (0%)	0 (0%)	12.57 (10.52-14.90)	Negative	23.09
Total	**92**	**92**	**0**	**0**	**12.50**	**Negative**	**23.75**
25 kGy: Fed only (Tank 1)	26	1 (3.9%)	7 (26.9%)	18 (69.2%)	35.88 (35.37-36.98)	Negative	21.21
25 kGy: Fed only (Tank 2)	22	0 (0%)	1 (4.6%)	21 (95.5%)	39.38	Negative	20.43
25 kGy: Fed only (Tank 3)	29	2 (6.9%)	4 (13.8%)	23 (79.3%)	32.95 (32.73-37.00)	Negative	21.07
Total	**77**	**3**	**12**	**62**	**36.07**	**Negative**	**20.90**
50 kGy: Inject and fed (Tank 1)	27	3 (11.1%)	7 (25.9%)	17 (63.0%)	35.15 (34.08-36.50)	Negative	21.80
50 kGy: Inject and fed (Tank 2)	30	3 (10.0%)	5 (16.7%)	22 (73.3%)	36.30 (35.39-36.76	Negative	21.95
50 kGy: Inject and fed (Tank 3)	26	4 (15.4%)	6 (23.1%)	16 (61.5%)	33.78 (31.16-41.83)	Negative	21.55
Total	**83**	**10**	**18**	**85**	**35.08**	**Negative**	**21.77**
50 kGy: Inject and fed (Tank 1)	27	23 (85.2%)	3 (11.1%)	4 (14.8%)	33.50 (31.76-35.66)	Negative	20.00
50 kGy: Inject and fed (Tank 2)	27	19 (70.4%)	6 (22.2%)	2 (7.4%)	33.72 (27.05-36.44)	Negative	20.09
50 kGy: Inject and fed (Tank 3)	28	22 (78.6%)	2 (7.1%)	4 (7.1%)	34.84 (33.06-36.60)	Negative	20.02
Total	**82**	**64**	**11**	**10**	**34.02**	**Negative**	**20.04**
50 kGy: Fed only (Tank 1)	24	0 (0%)	3 (12.5%)	21 (87.5%)	35.89 (34.54-36.60)	Negative	20.17
50 kGy: Fed only (Tank 2)	28	2 (7.1)	4 (14.3%)	22 (78.6%)	34.83 (32.94-41.48)	Negative	19.81
50 kGy: Fed only (Tank 3)	24	7 (29.2)	11 (45.8%)	6 (25.0%)	35.57 (33.54-36.68)	Negative	20.16
Total	**56**	**9**	**18**	**49**	**35.43**	**Negative**	**20.05**

#Positive result = both wells positive; Indeterminate result = one well positive + one well negative

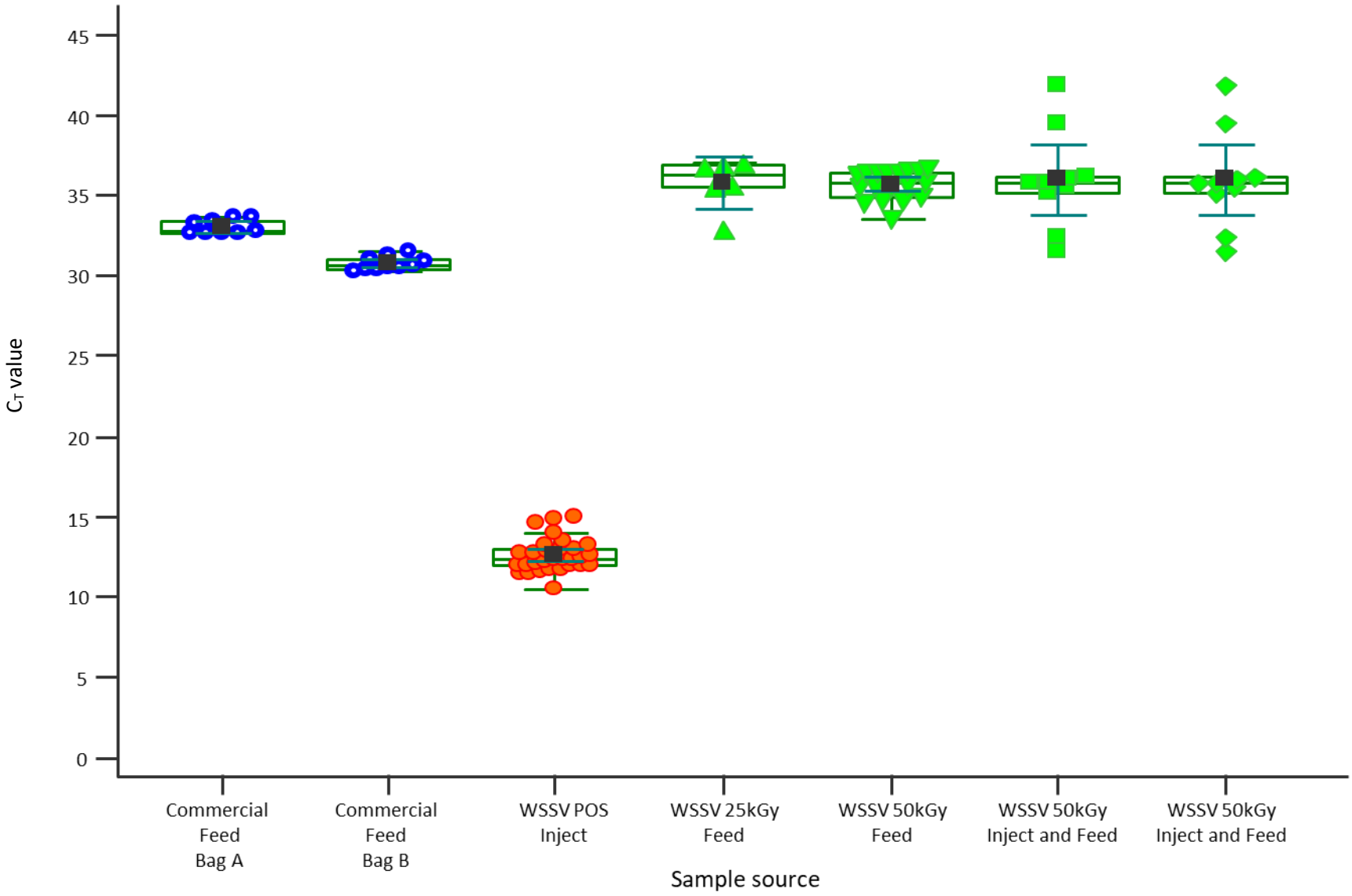


Figure 21. Graphical comparison of CSIRO WSSV qPCR results.

Table 17. Molecular test results after testing samples from Bag B

Sample	CSIRO WSSV qPCR	OIE WSSV qPCR	Artificial Probe qPCR	T4 Phage qPCR	Shrimp EF1 qPCR
Bag B: Top 01	31.57	32.13	Negative	27.05	33.21
Bag B: Top 02	30.41	31.38	Negative	26.98	32.45
Bag B: Top 03	30.88	32.05	Negative	27.07	32.36
Bag B: Middle 01	31.06	32.34	Negative	27.08	31.94
Bag B: Middle 02	30.35	31.63	Negative	27.03	32.18
Bag B: Middle 03	31.32	32.08	Negative	27.19	32.12
Bag B: Bottom 01	30.56	31.63	Negative	27.36	32.45
Bag B: Bottom 02	30.47	31.50	Negative	27.16	32.31
Bag B: Bottom 03	30.71	31.95	Negative	27.42	32.89
Bag B: Bottom 04	30.48	31.49	Negative	27.87	32.84
Mean	**30.78**	**31.82**		**27.22**	**32.48**
Range	**30.35 – 31.57**	**31.38 – 32.34**		**26.98 – 27.87**	**31.94 – 33.21**
NEC	Negative	Negative	Negative	27.25	Negative
NTC	Negative	Negative	Negative	Negative	Negative
POS Ctrl 1	23.43	Not tested	24.03	Negative	Negative
POS Ctrl 2	30.17	Not tested	30.82	Negative	Negative
POS Ctrl 3	Not tested	27.16	26.15	Negative	Negative
POS Ctrl 4	Not tested	34.23	33.07	Negative	Negative

NEC = Negative extraction control; NTC = No template control

2.2.2.3 Histological analysis of P. monodon

In all cases, only the cephalothorax of the prawn was examined via a mid-sagittal section, including the lymphoid organ, hepatopancreas, haematopoietic tissue, antennal gland, and upper digestive tract. A minimum of two sections were examined per animal.

There was no evidence of WSSV infection in any of the pre-trial samples nor negative control prawns.

All positive control prawns displayed histopathology consistent with WSSV infection, including widespread basophilic intranuclear inclusion bodies within tissues of ectodermal and mesodermal origin (gastric epithelium, epidermis, antennal gland, haematopoietic tissue and subepithelial connective tissue). Viral inclusions were also sometimes observed within the lymphoid organ. In addition, there was often diffuse necrosis of the lymphoid organ along with multifocal necrosis of the subcuticular epithelium of the stomach and epidermis of the integument.

There were no histopathological lesions, nor viral inclusion bodies consistent with WSSV, in any of the prawns exposed to gamma irradiated WSSV virus.

In the majority of prawns across all treatment groups, there were varying degrees of spheroid formation within the lymphoid organ. This is a non-specific change; however, it may be associated with an infectious process unrelated to WSSV infection. Specific histological findings are described in Table 18.

Table 18. Specific histological findings in prawns

Pre-trial samples

Prawn ID	Histopathology	WSSV inclusions
1	No significant findings	None
2	No significant findings	None
3	Lymphoid organ: Spheroid formation comprising 25% of the parenchyma with occasional apoptotic bodies.	None
4	No significant findings	None
5	Lymphoid organ: Spheroids comprising 50% of the parenchyma with moderate vacuolation, apoptosis and necrosis throughout and possible basophilic intracytoplasmic inclusions (Figure 22)	None
6	Lymphoid organ: Early spheroid comprising 50% of the parenchyma	None
7	No significant findings	None
8	Lymphoid organ: Spheroid formation comprising 50% of the parenchyma with possible basophilic intracytoplasmic inclusions	None
9	Lymphoid organ: Spheroid formation comprising 50% of the parenchyma	None
10	Lymphoid organ: Early spheroid formation comprising 25% of the parenchyma	None

Room C8: Negative Controls

Prawn ID	Histopathology	WSSV inclusions
Tank 1, Prawn 1	Lymphoid organ: Small numbers of early spheroids	None
Tank 1, Prawn 2	Lymphoid organ: Small numbers of early spheroids	None
Tank 2, Prawn 2	Lymphoid organ: Small numbers of early spheroids	None
Tank 2, Prawn 3	Lymphoid organ: Moderate numbers of spheroids with small numbers of apoptotic cells	None

Room C7: Positive Controls

Prawn ID	Histopathology	WSSV inclusions
Tank 1, Prawn 4	There is diffuse necrosis of the lymphoid organ and haematopoietic tissue with multifocal necrosis of the cuticular epidermis. INIBs are widespread in multiple tissues.	Positive
Tank 1, Prawn 5	There is diffuse necrosis of the lymphoid organ with spheroid formation and necrosis of the haematopoietic tissue. INIBs are widespread in multiple tissues.	Positive
Tank 2, Prawn 5	There is diffuse necrosis of the lymphoid organ and multifocal necrosis of the cuticular epidermis. INIBs are widespread in multiple tissues.	Positive
Tank 2, Prawn 6	There is diffuse necrosis of the lymphoid organ and in subepithelial connective tissue of multiple organs (Figure 23). INIBs are widespread in multiple tissues.	Positive
Tank 2, Prawn 7	There is mild, multifocal necrosis of the lymphoid organ with spheroid formation and multifocal necrosis of gastric subcuticular epithelium and connective tissue. INIBs are widespread in multiple tissues.	Positive
Tank 3, Prawn 7	There is mild, multifocal necrosis of the lymphoid organ with spheroid formation and multifocal necrosis of gastric subcuticular epithelium and connective tissue. INIBs are widespread in multiple tissues.	Positive
Tank 3, Prawn 8	There is diffuse spheroid formation within the lymphoid organ with necrosis of the surrounding connective tissue. There is multifocal necrosis of gastric subcuticular epithelium, connective tissue, epidermis and haematopoietic tissue. INIBs are widespread in multiple tissues.	Positive
Tank 3, Prawn 9	There is diffuse necrosis of the lymphoid organ and multifocal necrosis of the cuticular epidermis, subcuticular epithelium of the stomach and connective tissue. INIBs are widespread in multiple tissues.	Positive

Room C6: WSSV 25 kGy as feed

Prawn ID	Histopathology	WSSV inclusions
Tank 1, Prawn 1	Lymphoid organ: There is spheroid formation comprising 40% of the parenchyma	None
Tank 1, Prawn 2	There is a focal area of cuticle erosion and melanosis within the epithelium of the dorsal integument. Early spheroids comprise 40% of the lymphoid organ parenchyma	None
Tank 2, Prawn 1	Lymphoid organ: Spheroid formation comprises 30% of the parenchyma	None
Tank 2, Prawn 2	Lymphoid organ: Spheroid formation comprises 30% of the parenchyma with occasional apoptotic cells	None
Tank 3, Prawn 3	Lymphoid organ: Spheroid formation comprises 20% of the parenchyma	None
Tank 3, Prawn 4	No significant findings, only a small section of lymphoid organ available	None

Room C5: WSSV 50 kGy intra-muscular injection and feed exposure

Prawn ID	Histopathology	WSSV inclusions
Tank 1, Prawn 1	No significant findings	None
Tank 1, Prawn 2	No significant findings	None
Tank 2, Prawn 1	Lymphoid organ: Small numbers of spheroids Multifocal mineralised foci within the pereiopod subcuticular tissue.	None
Tank 2, Prawn 2	Lymphoid organ: Spheroid formation comprising 50% of the parenchyma	None
Tank 3, Prawn 1	No significant findings. No lymphoid organ is present.	None
Tank 3, Prawn 2	Lymphoid organ: Spheroid formation comprising 30% of the parenchyma	None
Tank 3, Prawn 3	Lymphoid organ: Small numbers of spheroids	None

Room C4: WSSV 50 kGy intra-muscular injection and feed exposure

Prawn ID	Histopathology	WSSV inclusions
Tank 1, Prawn 1	Lymphoid organ: Spheroid formation comprising 30% of the parenchyma	None
Tank 1, Prawn 2	Lymphoid organ: Spheroid formation comprising 30% of the parenchyma There is a small haemocyte cluster within the heart wall	None
Tank 2, Prawn 3	Lymphoid organ: Spheroid formation comprising 50% of the parenchyma	None
Tank 2, Prawn 4	Lymphoid organ: A single spheroid within the parenchyma	None
Tank 3, Prawn 1	Lymphoid organ: Spheroid formation comprising 40% of the parenchyma	None
Tank 3, Prawn 2	Lymphoid organ: Spheroid formation comprising 10% of the parenchyma There is a focal accumulation of degenerate cells within the hepatopancreas with central acellular basophilic material	None

Room C3: WSSV 50 kGy as feed

Prawn ID	Histopathology	WSSV inclusions
Tank 1, Prawn 2	Lymphoid organ: Spheroid formation comprising 20% of the parenchyma There is a single cluster of haemocytes within the heart wall	None
Tank 1, Prawn 3	Lymphoid organ: Spheroid formation comprising 20% of the parenchyma	None
Tank 2, Prawn 2	Lymphoid organ: Spheroid formation comprising 10% of the parenchyma	None
Tank 2, Prawn 3	Lymphoid organ: Spheroid formation comprising 50% of the parenchyma with moderate numbers of apoptotic cells	None
Tank 3, Prawn 1	Lymphoid organ: Spheroid formation comprising 10% of the parenchyma	None
Tank 3, Prawn 2	Lymphoid: Spheroid formation comprising 10% of the parenchyma	None
Tank 3, Prawn 3	Lymphoid organ: Spheroid formation comprising 20% of the parenchyma	None

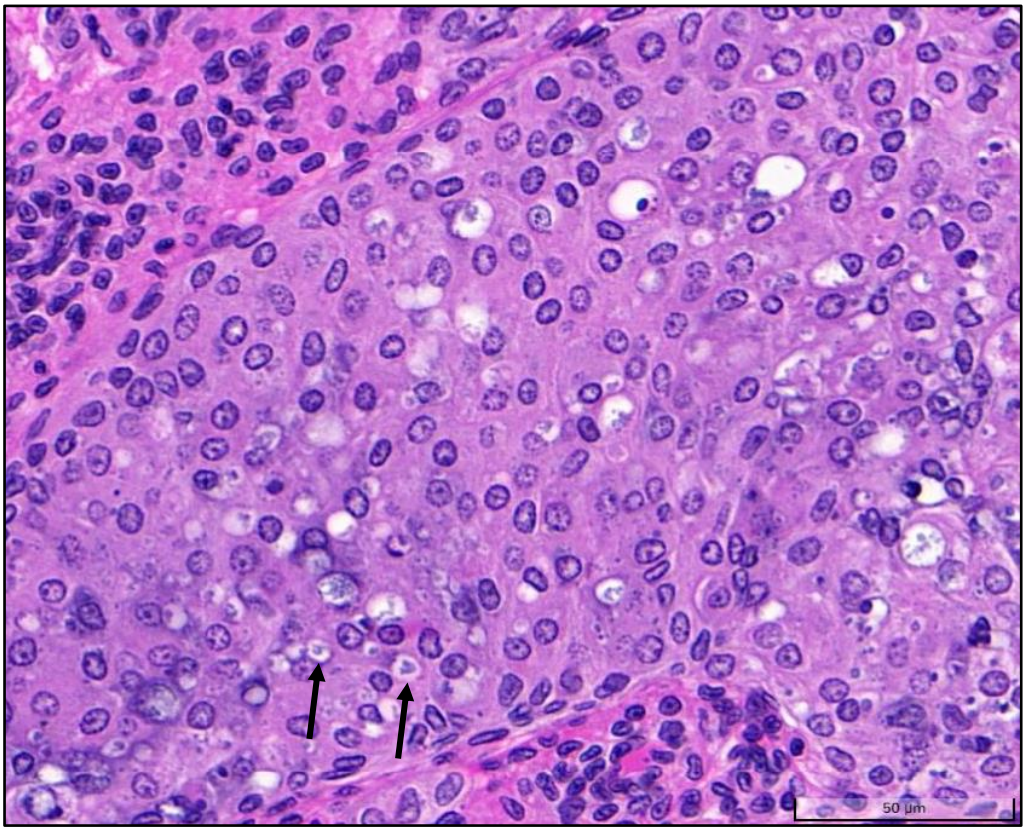


Figure 22. Lymphoid organ from Prawn 5 (pre-trial) showing spheroid formation, vacuolation, necrosis and possible cytoplasmic inclusions (arrows). Pre-trial prawn, number 5.

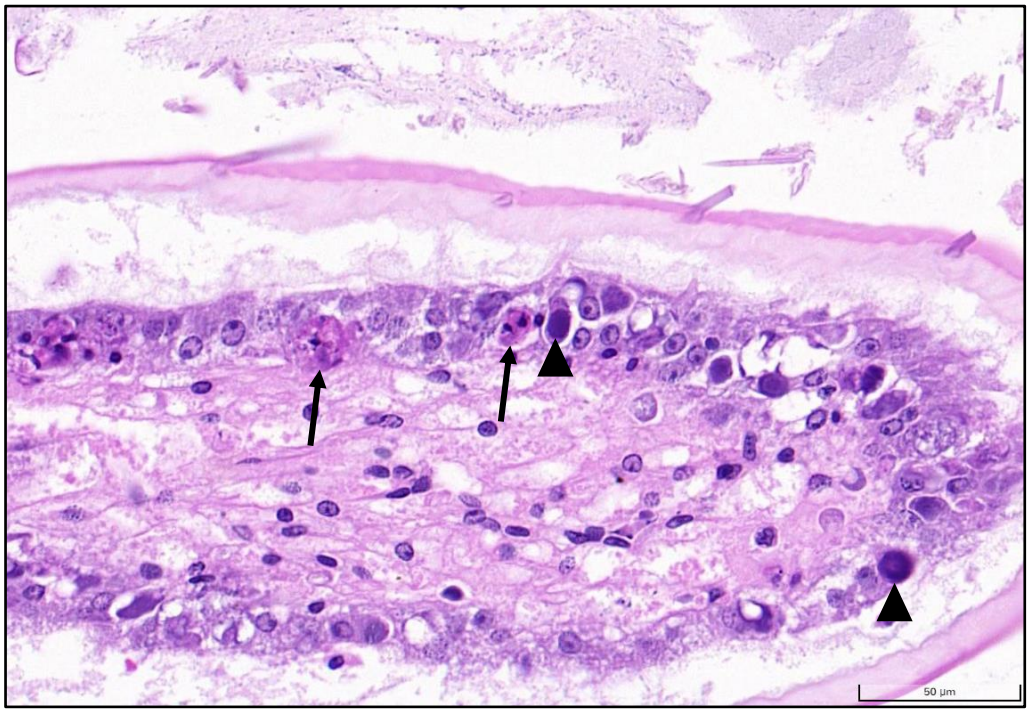


Figure 23. Subcuticular epithelium of the stomach showing epithelial cell necrosis (arrows) and intranuclear inclusion bodies (arrowheads). Positive control Tank 2, prawn 6.

2.3. Conclusions

After prawns were exposed to gamma irradiated WSSV-infected *P. monodon* tissue by either injection and feeding (50 kGy), or just feeding (25 kGy and 50 kGy), cumulative mortalities were low. Total mortality at the end of each trial was <4% and equivalent to the mortalities observed in negative control prawns. These results combined with an absence of pathological changes consistent with infection with WSSV, strongly suggests that gamma irradiation treatments of 25 kGy and 50 kGy completely inactivated the infectious WSSV present.

There were unexpected and inconsistent CSIRO WSSV qPCR positive test results in *P. monodon* surviving exposure to gamma irradiated material at the end of the 28 trial. These positive results may be due to low-level subclinical infection, residual WSSV circulating in the prawns (after injection and/or ingestion of non-infectious gamma irradiated WSSV-infected prawns) or residual WSSV DNA in the water and/or on surfaces in the tanks due to the introduction of gamma irradiated WSSV-infected prawns. The number of prawns testing positive by WSSV qPCR in different rooms was variable, particularly in the replicate 50 kGy inject and feed rooms, where 12% and 78% were test positive. This is unlikely to be due to the inoculum, where the WSSV qPCR C_T values were very similar across all treatments. A more likely source was the WSSV qPCR positive commercial prawn feed, as this was provided to the prawns daily for at least the last 21 days of the trial and the amount of food provided was more variable as it was at the discretion of the different staff maintaining the prawns in the different rooms.

Conclusion

The primary objective of this project was to determine whether gamma irradiation treatment of WSSV infected prawns at a dose lower than 50 kGy would inactivate the virus. The objective was further qualified by the bait wholesale industry who indicated that a reduction in treatment dose to 25 kGy or lower would be required to provide meaningful cost benefits.

The results from this project during Trial 1 have demonstrated that WSSV infected prawns, after gamma irradiation at 10 kGy, 15 kGy, 20 kGy and 25 kGy remain infective to healthy prawns when experimentally challenged via inoculation and feeding. The results from Trial 2, where prawns were exposed to gamma irradiated WSSV infected prawns (25 kGy) by feeding only, provided evidence that 25 kGy may inactivate WSSV at least to a level below that required to cause clinical disease, when exposed by a natural route of infection. However, the detection of non-viable WSSV nucleic acid in the commercial feeds used during trial 2, and the likelihood of feed being responsible for low level WSSV positive molecular test results in experimental animals across most treatments, needs to be resolved before any further consideration could be given to the level effectiveness of 25 kGy at inactivating WSSV in prawn tissue.

Implications

Trial 1 demonstrated that gamma irradiation of WSSV-infected prawns at 10 kGy, 15 kGy, 20 kGy and 25 kGy and exposure of P. monodon by injection and feeding failed to inactivate the infectious WSSV present. Based on results from Trial 1 and some uncertainty relating to the results of Trial 2 (unexpected WSSV positive test results in prawns surviving infection), wild prawns harvested within the WSSV movement restriction area for packaging and sale as fresh/frozen fishing bait should continue to be treated with gamma irradiation at a dose of 50 kGy.

Recommendations

While there were unexpected test results in prawns surviving exposure to gamma irradiated WSSV at the end of Trail 2, these were specific to trials involving WSSV. The experimental protocols developed during this project can be applied to other prawn pathogens if data regarding the effect of gamma irradiation on inactivation of infectious virus is required.

Further development

Due to the unexpected detection of WSSV nucleic acid in experimental animals during Trial 2, and the suggestion that these qPCR test results are due to contamination from non-viable WSSV nucleic acid in feed, Trial 2 should be repeated after a source of WSSV-negative feed has been identified. If this is not possible, consideration should be given to modifying the experimental design to include testing of samples less susceptible to surface contamination, such as hemolymph.

Two options to obtain WSSV-negative feed for future trials include:

1. The use of wild caught green prawns known to be free of WSSV (e.g. Northern Prawn Fishery) that have been gamma irradiated to render them non-infectious to other pathogens they may be carrying and feed these to the prawns for the duration of the trial.

2. Batch testing of commercially produced prawn feed prior to commencement of trial to ensure WSSV nucleic acid is not present.

Extension and Adoption

After the completion of Trial 1, results were presented to industry members, scientific colleagues, representatives from FRDC, representatives from Department of Agriculture and Fisheries, representatives from Department of Agriculture, Water and the Environment (including the Australian Chief Veterinary Officer) where the project modification described for Trial 2 were agreed.

An AQUAPLAN Webinar is planned when the Final Report has been approved.

Due to the unexpected WSSV-positive test results in *P. monodon* surviving at the end of Trial 2, unless Trial 2 is repeated with WSSV-free prawn feed, the authors do not believe the research is worthy of publication in a peer-reviewed journal.

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Appendices

Appendix 1. List of Project Staff

Name	Position	Organisation
Dr Stephen Wesche	Principal Scientist	Biosecurity Queensland, Department of Agriculture and Fisheries
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Dr Ian Anderson	Principal Veterinarian (Aquatic Pathology)	Biosecurity Queensland, Department of Agriculture and Fisheries
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Dr Mark Crane	Senior Principal Research Scientist	CSIRO ACDP Fish Diseases Laboratory
Dr David Cummins	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Lynette Williams	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Joanne Slater	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Dr Serge Corbeil	Senior Research Scientist	CSIRO ACDP Fish Diseases Laboratory
Mr John Hoad	Senior Experimental Scientist	CSIRO ACDP Fish Diseases Laboratory
Mr Reuben Klein	Research Technician	CSIRO ACDP Fish Diseases Laboratory
Ms Stacey Valdeter	Research Technician	CSIRO ACDP Fish Diseases Laboratory