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FINAL REPORT

Investigating emerging causes of disease in a commercial Australian prawn farm

A prospective trial and epidemiological data review

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Investigating emerging causes of disease in Australian Prawn Farms: A prospective trial and epidemiological data review

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Abbreviations

APF – Australian Prawn Farms

FFVS – Future Fisheries Veterinary Service

UofA - University of Adelaide

BSL – Queensland Biosecurity Sciences Laboratory

PRF - Pacific Reef Fisheries

PL – Post Larvae

TSV - Taura Syndrome Virus

WSSV - White Spot Syndrome Virus

YHV 1 - Yellow head virus (Genotype 1)

YHV 7 - Yellow head virus (Genotype 7)

GAV – Gill Associated Virus

AHPND - Acute Hepatopancreatic Necrosis Disease

CMNV - Covert mortality nodavirus

EHP - Enterocytozoon hepatopenaei

NHP - Necrotising hepatopancreatitis bacterium

IHHNV - Infectious hypodermal and hematopoietic necrosis

IMV - Infectious Myonecrosis Virus

MoV - Mourilyan virus

LsV - Laem-Singh Virus

When 2 - Wenzhou shrimp virus-2

HDV - Decapod Hepandensovirus 1

PVNV - Penaeus vannamei nodavirus

PPCPs - Personal care products

CT – Cycle Threshold

PCR - Polymerase chain reaction

NGS - Next-generation sequencing

TCBS - Thiosulfate-citrate-bile salts-sucrose agar

PBS - Phosphate Buffered Saline

NH_3 – Unionised ammonia

TAN – Total ammonia nitrogen

QA – Quality Assessment

QC – Quality Control

SHIV - Shrimp Haemocyte Iridescent Virus

Executive Summary

This project was developed by Future Fisheries Veterinary Service (FFVS) in collaboration with Australian Prawn Farms (APF), James Cook University (JCU) Aqua Path laboratory, Queensland Biosecurity Sciences Laboratory (BSL) and the University of Adelaide (UofA). Unprecedented mortality events occurred in a commercial Australian prawn industry hatchery and grow-out farm between August 2018 and January 2019. Initial investigations did not identify the consistent involvement of an infectious agent in particular. A range of potential risk factors appeared to be associated with tank- and pond-level mortality events, however subsequent mitigation of these in the hatchery, did not appear to fully resolve the mortalities. The chronology of outbreaks within the hatchery and soon after pond stocking raised alarm that this could be an Australian case of Early Mortality Syndrome. Further investigation was warranted to identify if emerging infectious agents may be involved and to determine evidence-based control and prevention methods accordingly. During 2019, a prospective grow-out pond trial and retrospective epidemiological analysis of farm data were performed, to identify potential causative agents and/or risk factors associated with the mortality events. The project outputs will help shape recommendations for reducing disease risks, improve hatchery and grow-out farm practices, and further refine disease investigation approaches. The project was funded through the Fisheries Research and Development Corporation (FRDC).

Background

The Australian prawn farming industry is a rapidly growing component of the aquaculture sector, producing 4,264 tonnes of edible prawns at value of $85.7 million during 2016-17 (Mobsby, 2018). In late 2018, a leading producer, suffered unprecedented mortality events within the hatchery and subsequently during grow-out pond production. Complete loss of over half of its hatchery tank populations and grow-out pond populations occurred, with total cumulative mortality estimation in excess of 200 million animals. The magnitude and presentation of these mortalities is uncommon across the Australian prawn farming industry and directly threatened to sustainability of the farm.

Mortality within the hatchery occurred throughout all post-larvae stages, whereas in grow-out ponds prawns failed to materialise at the 6-8-week check suggesting an early post stocking mortality. Investigations performed during the mortality events failed to identify a definitive infectious cause. This project was developed from the need to better understand the range of potential trigger(s) and determining factors that led to the mortality events in both the hatchery and grow-out. The use of modern analytical tools, such as Next Generation Sequencing (NGS), were employed to investigate of the presence of emerging infectious agent(s), and a retrospective epidemiological analysis of farm data was undertaken to identify potential risk factors associated with the mortality events. Understanding the cause(s) of the unprecedented mortality event is likely to be of value to all farms across the prawn farming industry, which may encounter similar events in the future.

Objectives

1. Explore the presence of potential infectious agents in six batches of preserved post-larvae (PL) from affected APF hatchery tanks from 2018 season
2. Develop a quantitative PCR (qPCR) for bacterial toxin genes selected from the NGS analysis of bacterial isolates cultured from APF hatchery water samples collected during 2018 season
3. Detect the presence of the selected bacterial toxin genes in prawn PL samples from affected APF hatchery tanks during 2018 season
4. Develop fit-for-purpose grow-out pond sampling methods to monitor stock health status
5. Longitudinal sampling of three trial grow-out ponds to archive chronological specimens for subsequent investigation if warranted by sub-standard survival at 6-8 weeks.

6. Investigate the cause(s) and determinants of sub-standard survival in trial grow-out ponds, should it occur, using range of diagnostic tests and epidemiological assessment of risk factors in 2019
7. Measure levels of agri-chemical pollutants in trial grow-out ponds water and consider their effects on prawn larval health

Methodology

Methods for disease investigation in commercial prawn farms were assessed. NGS was performed to explore the presence of potential emerging pathogens and corresponding qPCR were developed for the three selected bacterial toxins; YaFO, RtX, and zon occludens (also referred to as ZOT). Further testing of archived hatchery PL samples was conducted to determine the potential significance of the identified toxin genes. A prospective trial was conducted in January 2019 using three commercial grow-out ponds to allow for multidisciplinary diagnostic testing of purposively collected samples to be performed, should mortality re-occur in these ponds. Finally, an epidemiological analysis of hatchery and grow-out farm (stocking and mortality records) as well as test data was performed to identify potential risk factors associated with mortality events encountered during 2018/19 commercial production.

Results/key findings

The NGS exploration identified three bacterial toxin genes of interest (i.e. YaFO, RtX, and zon occludens). Subsequently, qPCR assays were developed to detect and quantify for each toxin gene respectively. All three assays revealed highly repeatable quantification (i.e. small variability between replicates within a specimen) supporting appropriate QA/QC.

The presence and the amount of the three toxin genes could not be associated with the hatchery or grow-out stage mortalities. No other infectious organism (i.e. virus, bacteria, fungi or parasite) could be identified as a likely cause of the mortality events either in the hatchery or grow-out stage of production.

The analytical results available from retrospective cases in ponds were not from samples collected at the optimal time for investigation, hence it is possible a pathogen may have been missed.

However, some factors were identified with differing strengths of association with mortality events, in both the hatchery and grow-out stage of production. In the hatchery, tanks (i) stocked with ≥113 nauplii L^{-1}, (ii) merged with another tank or that (iii) did not received a pre-emptive course of oxytetracycline hydrochloride treatment were significantly more likely to be culled. During the grow-out stage, ponds (i) stocked PL <11 days after filling, (ii) with elevated levels of the *Nitzschia* spp. algae 30 days post stocking or (iii) with a minimum salinity <36 ppt during the 6 weeks post stocking were significantly more likely to experienced 100% mortality.

A lack of further defining characteristics of mortality events encountered in the hatchery and grow-out stages limited the epidemiological case definition to mortality alone (defined at culled population, 0% survival). In the grow-out stage, data was only available from 47 populations, which may have limited the significant risk factors that were able to be identified in the analysis.

None of the three trial ponds experienced the 100% mortalities experienced the previous season on the farm. At harvest (111-118 days post stocking) the estimated adjusted survival for each trial pond was 22.83% (2065 Kg), 97.80% (7572 Kg) and 29.32% (2655 Kg), for pond 43, 44 and 45, respectively. Farm staff successfully demonstrated regular and reliable capture of post stocked free ranging PL using feed trays every 2-3 days for the duration of the trial. Negligible levels of agri-chemical pollutants were detected by passive water sampling devices and, in this case, were not found to be associated with elevated mortality. Histopathological investigation of the first two weeks of the trial did not identify any pathology that could explain the observed survival differences across the three trial ponds. The trial pond with highest survival (#44) originated from a different hatchery tank population to the other two. Further histopathological analysis was not possible due to unanticipated tissue autolysis in samples.

Implications for relevant stakeholders

This project demonstrated the use of a veterinary disease investigation framework in an Australian prawn farm. This approach could be replicated throughout the industry, to assist future disease incidents. We demonstrated how NGS could be used to screen for potential pathogens not readily detectable by common diagnostic modalities, and to develop targeted diagnostic tests.

The retrospective epidemiological analysis of farm records in complement of the laboratory findings provide valuable cues during a disease investigation and help shape evidence-based recommendations to adapt hatchery and grow-out farm practices and may yield increased benefits with wider industry adoption.

The industry acceptance that 'PL growth and health monitoring immediately following stocking into an earthen pond is not feasible' was resolved. However, the capture and assessment of larvae, in this case, was not able to predict the survival outcome and determine the cause for the observed survival difference. This approach, after further validation, could enable health investigation and shorten response times and permit alternative management arrangements, such as restocking, where survival can be determined to be poor. These could have significant production benefits to industry with further validation and data generation.

The presence of agri-chemicals pollutants within a commercial aquaculture production system was highlighted, furthering previous industry research, and demonstrating methods to monitor these interactions with passive sampler devices.

We provided further guidance to optimise disease investigation on PL, including sample collection and processing for molecular biology, microbiology and histopathology. Consideration of the proposed approaches will increase the output quality and value of the testing findings.

Recommendations

An extensive list of recommendations aimed to reduce the probability of future related mortality in hatchery and grow-out production are detailed in the report. We also provide comments on the project limitations and improvements to diagnostic processes. Further review of data collected from the upcoming production season may assist strengthening or weakening of the significance and probability of the identified risk factors. In addition, where new issues are identified in the hatchery or grow-out stage an investigative framework has been established to allow for improvements to sample collection, processing, analysis and results interpretation.

Keywords

Black Tiger Prawn (*Penaeus monodon*), Veterinary Disease Investigation, Epidemiology, Molecular Biology, Histopathology, Prospective Trial, Vibriosis, Hepatopancreatitis, qPCR, Toxin Genes.

1. Introduction

Hatchery outbreak

Australian Prawn Farms Pty Ltd (APF) has been a leading producer of farmed prawns in Australia for many years. The APF hatchery suffered unprecedented mortality events within its hatchery tanks (68 larval rearing tanks) during the 2018 production period (July-November). Future Fisheries Veterinary Service Pty Ltd (FFVS) and Infiniseas Co. (Daniel Gruenberg) were invited to investigate. The farm sought molecular biology diagnostic testing services of AquaPath laboratory at James Cook University (JCU) and the veterinary laboratory diagnostic services of Queensland Government Biosecurity Science Laboratory (BSL) throughout the investigation.

Investigations commencing on the 26th September 2018, identified potential husbandry, water quality, nutritional and parasitic stressors associated with mortality. The correction of these factors in subsequent hatchery runs (Run 2-3), did not resolve the hatchery mortality issue. Pathology results from larval tanks experiencing mortality during this period (Run 2-3) were suggestive of a *Vibrio*-based hepatopancreatitis and enteritis overwhelming compromised larvae, however a definitive diagnosis of an infectious disease was not reached. The use of off-label Oxytetracycline via veterinary prescription was utilised as a clinical trial as a presumptive control from October onwards (Run 3-4). Hatchery production immediately improved and thereby permitted hatchery production of sufficient post-larvae (PL) to stock the farm ponds in November with hatchery tanks from run 3 and 4.

Grow-out pond outbreak

In early December, 5-6 weeks following stocking of ponds using run 3 and 4 PL from farm's hatchery, it was noticed a significant proportion of grow-out ponds had experienced mortalities approaching 100% in 27 of 47 (57%) of grow-out ponds stocked (see appendix 1 Table 15). This was unprecedented in the farm's experience. Unfortunately, prawn and environmental samples from the period prior to detection of mass mortality were not available to facilitate a more detailed investigation into the mortalities, when the prawns were being lost.

Further investigation - Investigating emerging causes of disease in a commercial Australian prawn farm

This project was discussed with experts from APF, JCU, Australian Animal Health Laboratories (AAHL), BSL and the Australian Prawn Farmers Association (APFA), to determine a framework which provided an opportunity to better understand the losses experienced in 2018 by APF and determine if pathogen(s) of national industry importance were involved. The project and its framework were funded by the Fisheries Research and Development Corporation (FRDC) to help protect the prawn farming industry should a similar event of severity and scale occur in subsequent seasons on this or other prawn farms.

Bacterial cultures from sick PL's from affected hatchery tanks did not reveal a consistent single pathogen species across the multiple affected hatchery tanks. Diagnostics testing performed during the hatchery mortalities was not extensive, with a deficiency in bacterial cultures from affected post larvae. Understanding the cause of the substantial hatchery PL mortality is of value to all hatcheries across the prawn farming industry. Intermittent individual tank mortality events are reported at most prawn hatcheries at some point over the history of their operation. With use of new diagnostic tools, such as NGS, exploration for novel pathogens can be commenced using stored material from historical hatchery outbreaks. Further investigation, through this project, of hatchery and pond mortalities, offers the opportunity to better define the risk factors associated with the syndrome and inform possible control and prevention measures.

Farm management plans can incorporate the control and prevention measures identified in this project, to reduce the risk of similar events occurring in future seasons at this farm and others in the prawn farming industry.

2. Objectives

1. Describe potential pathogens in six batches of preserved PL's from sick APF hatchery tanks from 2018 season
2. Develop qPCR for bacterial toxin genes identified in NGS analysis of bacterial cultures collected from APF hatchery water during 2018 season
3. Determine the presence of putative bacterial toxins in prawn larvae samples from sick APF larvae tanks during 2018 season
4. Identify useful post-stocking sampling methods for PL's to monitor health status
5. Preservation of samples from three trial stocking ponds to provide temporally relevant samples for subsequent analysis if warranted by low survival determination at 5-8 weeks.
6. Determine the cause(s) of low survival in experimental prawn ponds, should it occur, using range of diagnostic tests and epidemiological assessment of risk factors
7. Levels of agri-chemical pollutants in source water entering ponds will be measured and their effects on prawn larval health considered

3. Method

3.1. Project Management

The project was managed by the Principal Investigator, Matt Landos (Future Fisheries Veterinary Service) in consultation with the project steering committee comprised of: Matt West (Australian Prawn Farms); Kelly Condon (James Cook University); Ian Anderson (Qld Govt Biosecurity Sciences Lab); Charles Caraguel (University of Adelaide) with Wayne Hutchison (FRDC) observer.

This project involved multiple diagnostic and investigative aspects to meet the project objectives. See Table 1 below for more details as to how each project aspect relates to the objectives.

Table 1. Project objectives and related sections covered in the report

	Project Objectives	***Related Methods Sections***	***Related Results Sections***
1	Describe potential pathogens in six batches of preserved PL's from sick APF hatchery tanks from 2018 season.	3.1.; 3.2.; 3.3.	4.4; 4.1; 4.3
2	Develop qPCR for bacterial toxin genes identified in NGS analysis of bacterial cultures collected from APF hatchery water during 2018 season.	3.1.; 3.2.	4.2
3	Determine the presence of putative bacterial toxins in prawn larvae samples from sick APF larvae tanks during 2018 season.	3.1.; 3.2.; 3.3.	4.3; 4.2; 4.1
4	Identify useful post-stocking sampling methods for PL's to monitor health status.	3.1.; 3.4.	4.6; 4.5
5	Preservation of samples from three trial stocking ponds to provide temporally relevant samples for subsequent analysis if warranted by low survival determination at 5-8 weeks.	3.1.; 3.3.; 3.4.	4.5
6	Determine the cause(s) of low survival in experimental prawn ponds, should it occur, using range of diagnostic tests and epidemiological assessment of risk factors.	3.1.; 3.2.; 3.3.; 3.4.	4.5; 4.5; 4.6; 4.1
7	Levels of agri-chemical pollutants in source water entering ponds will be measured and their effects on prawn larval health considered.	3.1.; 3.4.	4.7

3.2. Further Testing of Archived Post Larvae (PL) Samples

Sample collection

Archival samples of 135 routine pre-stocking hatchery PL samples were available for further molecular biology testing. These included PL populations from run 1 (n=52), run 3 (n=27), and run 4 (n=56), in addition to samples collected in the prospective trial ponds.

Methodology used to collect these samples differed between the archived samples.

1. Collection of routine pre-stocking hatchery samples involved capture of PL, counting of 100-120 PL into a specimen container containing of lysis buffer (approximately 1mL tissue to 4mL lysis buffer).
2. Collection of opportunistic hatchery samples from tanks experiencing elevated mortality involved capture of PL, using a dip-net ‘scoop’ into specimen container of lysis buffer (approximately 1mL tissue to 4mL lysis buffer).
3. Collection of prospective trial samples was as described in the section below.

All population samples were sent to JCU Aquapath lab for further processing and testing. All population samples were homogenised before extraction as five separate replicates for subsequent molecular biology testing.

Next generation sequencing (NGS)

To explore for the presence of common potential pathogens across the groups of affected populations Next generation sequencing (NGS) was performed, externally to this project, on hatchery PL samples collected from multiple run 3 larval rearing tanks experiencing elevated mortality at the time of sampling in October 2018. Tissues types used in this testing included;

1. Mixed bacterial isolates cultured of homogenised whole post larvae and culture tank water from hatchery tanks experiencing elevated mortality (Tanks 27, 30, 36 and 38), collected on 15/10/18, using an erythromycin infused TCBS agar plate.

Toxin gene qPCR development

YaFO, Rtx and zon occludens toxin genes identified using NGS, were used to develop qPCR tests to facilitate further investigate the potential involvement of these toxin genes in hatchery and grow-out mortality using archived routine samples of hatchery PL tanks and in samples collected in the prospective trial.

The repeatability of these diagnostic tests was briefly assessed through the epidemiological analysis.

3.3. Epidemiology Data Analysis

Case definition

A case definition of 0% survival (or 100% mortality) was used as inclusion criteria to classify hatchery tanks and/or grow-out ponds as a ‘case’ population.

A case definition of greater than 0% survival (or less than 100% mortality) was used as inclusion criteria to classify hatchery tanks and/or grow-out ponds as a ‘non-case’ population.

> *This does not imply that survival marginally greater than zero is not reflective of significant mortality but is a necessary data sorting step in order to allow appropriate statistical analysis*.

Data analysis

Analysis of available data from the 2018/19 hatchery production and grow-out pond stockings was undertaken to identify associations between potential contributory risk factors to mortality events in ponds and tanks. Appropriate statistical methods such as Linear Regression Analysis, Logistic Regression, Mixed Effects Logistic Regression, Pearson’s Correlation were utilised.

Data were reported in odds ratio and probability with 95% confidence interval and p-value provided.

Data outputs were repeated to ensure integrity and compiled using Microsoft Excel.

A tiered approach was used to report data results from the highest probability of association (highest level of data scrutiny and statistical significance determined, 10) to lowest probability of association (lowest level of data scrutiny and statistical significance determined, 0). This allowed for a scaling of data outputs and subsequent recommendations to direct farm investment to variables with a stronger or more significant association and therefore potential benefit of mitigation.

Where gaps in the data set were identified those populations/variables/data points were excluded from analyses.

Table 2. Significance Classification for data outputs based on the apparent strength of association between a given variable (exposure) and the case (outcome: zero survival). Reflective of the level of analysis and significance maintained throughout. Scale 10 (highest level of significance) the 1 (lowest level of significance), no significance (0).

Identified Risk Factor Analysis - Classification Level of Significance	
10	Comparative variable mixed effects logistic regression analysis (Significant), Independent variable mixed effects logistic regression analysis (Significant), Logistic regression or linear regression analysis (Significant). High collinearity and confounding factors impact not detected.
9	Comparative variable mixed effects logistic regression analysis (Not Significant), Independent variable mixed effects logistic regression analysis (Significant), Logistic regression or linear regression analysis (Significant). High collinearity and confounding factors impact not detected.
8	Comparative variable mixed effects logistic regression analysis (Not Significant), Independent variable mixed effects logistic regression analysis (Significant), Logistic regression or linear regression analysis (Significant). High collinearity and confounding factors impacts were detected.
7	Comparative variable mixed effects logistic regression analysis (Not Significant), Independent variable mixed effects logistic regression analysis (Not Significant), Logistic regression or linear regression analysis (Significant).
6	Logistic regression or linear regression analysis (Significant).
5	Logistic regression or linear regression analysis (Not Significant)
4	Single variable difference - case vs non-case populations (Significant - Outside standard error).
3	Single variable difference - case vs non-case populations (Not Significant - Within standard error).
2	Single variable difference between case populations and literature
1	General anomaly identified
0	No significance identified

3.4. Prospective Grow-out Trial

Site/pond selection and preparation

Three commercial grow-out ponds which had already experienced complete mortality (0% survival, case ponds) in December following stocking of PL from APF hatchery runs 3 and 4, were selected for use in the prospective grow-out trial. The three prospective grow-out trial were completely drained, prepared for re-stocking, filled and fertilised via management practices that as closely as possible reflected the approach used during the 2018/19 commercial grow-out season, where elevated mortality was experienced.

Molasses and Silica added where required throughout the trial (as per typical APF protocol).

Animal recruitment

Post larvae from the APF hatchery were not available for the pond trial, so PLs used were purchased from Pacific Reef Fisheries (PRF) hatchery in Guthalungra QLD. Three 3000L transporter tankers were used for transport of PL to the APF Ilbilbie grow-out pond farm. Approximately 400,000 PL were stocked into each of the three grow-out ponds on 10 January 2019.

Observations and sample collection

Diagnostic samples were collected from each pond population at the following post stocking day timepoints of the trial: 0 (pre-stocking), 4, 6, 8, 11, 13, 15, 18, 20, 22, 25, 27, 29, 32, 34, 36, 39, 41, 43, 46, 48, 50, 53 (n=23 per ponds; 69 timepoints total).

Additional feed trays were placed into each pond (3-6 feed trays in each pond) to facilitate capture of PLs for diagnostics, post stocking. As a safeguard, in the event PLs were not able to be recovered from feed trays, each pond had three separate small enclosure pens built and installed into each pond, ensuring access to PLs throughout the trial. The pens were approximately 1 x 2 x 1 metre (width x length x depth) and were each stocked with approximately 2000 PL (See Appendix 1).

Routine sample collection involved:

1) Histopathology
    a. An estimated 50-100 prawns from each of the prospective trial ponds were captured from feed trays and transported in 20L plastic buckets to the farm laboratory where they were placed into fixative (Davidson's solution for 24 hrs then transferred to 70% ethanol).
    b. Once prawn grew to >40mm in length they were injected with Davidson's fixative prior to placement into the fixative solution, additionally from this point in the trial fewer animals were sampled (30-50 prawns).
    c. Samples were stored at room temperature until analysis.
2) Molecular biology
    a. Prawns were sampled whole into sterile PBS at a ratio of 1 tissue: 1 PBS.
    b. Multiple sterile stainless-steel beads were used to homogenise the prawn/PBS sample.
    c. 1mL of homogenised sample was placed into 4mL of Lysis buffer.
    d. Samples stored at room temperature until analysis
3) Gross and microscopy assessment of prawns
    a. Count the number of dead prawns per tray
    b. Count the number of living prawns per tray (from day 20, 30/01/19, onwards)
    c. Measure average length (mm) and Average weight (g) (from day 22, 01/02/19, onwards)
    d. Prawn Health Assessment (See Table 3, below).

Water quality monitoring and pond management

Pond water quality was monitored routinely in all three ponds, consisting of twice daily (DO, pH, water temperature), once daily (TAN), every 3 days (algae species and relative abundance), weekly (alkalinity, salinity). Pond management was as per normal APF practices, including triggers for addition of molasses, silica and adjustments to water exchange and paddlewheel use.

Table 3. Prawn Health Assessment framework used during the prospective trial. Adapted from (FAO Fisheries and Aquaculture Department, 2007)

Category	***Method***	***Score - 0***	***Score - 5***	***Score - 10***
Stress Test	Place prawns into an aerated container with 50% ambient pond salinity for at least 1 hour. Sample 50-100 when small; 20-50 when larger. Performed only once per week (each Friday).	>10% Mortality	5-10% Mortality	<5% Mortality
Swimming Activity	Visual assessment of approximately 5-10 prawns	<75% Active	75% Active	Most Active
Size Variation	Visual assessment of approximately 5-10 prawns	<70% same stage/size	80-70% same stage/ size	80%+ same stage/size
Morphology/ Deformity	Visual assessment of approximately 5-10 prawns	Obvious deformity in many prawns	Very mild in few prawn's deformity	No deformity present
Fouling	Visual assessment of approximately 5-10 prawns. Assessing for protozoa, bacteria, fungi, algae, dirt.	heavy fouling	medium fouling	no fouling
Intestine	Visual and microscopic assessment of approximately 5-10 prawns	light/ no vacuoles or peristalsis	Partially full/ some vacuoles and peristalsis	Full/ Vacuoles/ Peristalsis
Muscle: Gut Ratio	Visual and microscopic assessment of approximately 5-10 prawns	muscle <50% width	muscle 50-75% width	muscle >75% width
Hepatopancreas	Visual and microscopic assessment of approximately 5-10 prawns	light/ empty/ no vacuoles	Partially dark and full/ some vacuoles	Full/ Dark/ Vacuoles
Gills	Visual and microscopic assessment of approximately 5-10 prawns	heavy fouling and melanin	medium fouling/ scant melanin	no fouling/no melanin

To investigate the potential role of a range of urban and agriculture origin chemical contaminants (pharmaceuticals and personal care products, PPCPs; and neonicotinoid compounds) two replicate empore disc (ED) passive samplers were deployed into one of the three trial ponds. Passive samplers suspended mid-water column attached to a metal stake and float approximately 5 meters away from the pond edge. The samplers were retained in the pond until the approximate mid-point of the trial, then removed and replaced with new samplers.

Trial length

The prospective trial ran for 56 days following stocking (day 0, 10/01/19).

Prawn sample testing

A steering committee meeting was held on 22/03/19 following commencement of the trial to further discuss histopathology and molecular biology diagnostic testing to be performed from the samples

collected from prospective trial ponds based on the estimated survival in each of the three populations at 8 weeks post-stocking or the conclusion of the trial.

4. Results

Molecular biology and histopathology testing were performed on a range routine and opportunistic samples collected from the 2018/19 commercial hatchery and grow-out production and grow-out trial pond populations. An epidemiological data analysis was performed using the diagnostics testing results and existing, relevant farm data sets. The results are displayed in a format to best align with the project objectives, with the exception of the epidemiology analysis which spans across multiple aspects of the project objectives and is covered in a separate section.

4.1. Epidemiological analysis of risk factors associated with low survival in the APF 2018/19 commercial hatchery, commercial grow-out pond, and prospective trial grow-out pond populations.

All the available farm data that was used for the epidemiological analysis was collected by APF hatchery staff (Tony Charles and Ryan Lowrey), grow-out staff (Matt West and Andrew Smith), and prospective trial staff (Skye Tara Lewis). Initial data analysis was performed by FFVS, with subsequent sophisticated data analysis performed under guidance of the project epidemiologist, Dr Charles Caraguel, at the University of Adelaide (UofA), Roseworthy Campus and at the FFVS office.

This data was collated and cleaned using Microsoft Excel, statistical data analysis to occur using STATA v15.1.

Data analysis was performed in a stepwise fashion seeking to eliminate weaker and not significant associations from significance, while stronger and significant associations were rigorously tested for relative significance of each association. Additional data was generated from the primary data set to change continuous format variables (e.g. 12%, 27%, 76%, etc) into a binary format (e.g. 0, 1), for some analysis (e.g. logistic regression). For other analysis raw data was kept in a continuous format for analysis (e.g. linear regression).

Comparative data analysis was performed using a range of analyses to explore strength and significance of any association with case definition (0% survival). A range of differing case definitions were briefly explored. However, 0% survival was considered the most appropriate for assessment of the data sets (Charles Caraguel Pers. Comm.).

The strength of the associations between potential risk factors (exposure) and the case definition (0% survival) (outcome) is reported using odds ratio (see below and Appendix 2 and 3) and odds ratio (OR). The odds reflect the probability of an event occurring to that of it not occurring. The odds ratios reflect the ratio of two odds. This is an epidemiological measure of the strength of association. The further the OR is above zero the greater the strength of the association between the exposure and the outcome (e.g. case), whereas the further the OR is below zero the greater the strength of the association between the exposure and the non-outcome (e.g. control).

4.1.1. Hatchery

Detailed data analysis using STATA v15.1 was performed on 40 different potential risk factor variables within the hatchery to explore strength of association with case definition (culled, 0% survival). See full detailed results in Appendix 1 and 2.

High Probability Risk Factors

A strong association between the following variables and being culled, remained highly significant ($p<0.01$) throughout all analysis and comparisons performed;

1. Merging of hatchery tanks

- The odds of a hatchery tank that was merged to be culled was 30.03 times greater than the odds of a non-merged tank (highly significant, $p<0.001$).

2. Stocking larval rearing tanks with 900,000 or more nauplii (8000L tanks, equivalent to 112.5 PL per litre).
    - The odds of a hatchery tank initially stocked with 900,000 nauplii or more to be culled was 13.37 times greater (highly significant, $p<0.02$), than the odds of a hatchery tank stocked with less than 900,000 nauplii.
3. Not performing in water treatment of larval rearing tanks with Oxytetracycline hydrochloride (20 mg/L) performed at least once.
    - Treating hatchery tanks with oxytetracycline hydrochloride at least once reduced the odds to be culled by 99.9% (highly significant, $p<0.001$), compared to not treating.

Low Probability Risk Factors

An association between the following variables and the being culled, was found to be significant ($p<0.05$) using mixed effects logistic regression analysis. However, no longer remained significant ($p>0.05$) when analysing alongside other significant variables due to correlation or collinearity interactions;

1. Not performing in water treatment of larval rearing tanks with Formalin (5-10 mg/L) performed at least once, or more than once, or twice.
    - Hatchery tanks that had any, or just one, or two Formalin treatment during larval rearing had apparent reduced odds to be culled.
2. Not being from hatchery run 4 or 3.
    - Hatchery tanks from run 3 or 4 had apparent reduced odds to be culled.
3. Not being from larval rearing tanks in rearing room 1 or being from rearing room 3.
    - Hatchery tanks from rearing room 1 had apparent increased odds to be culled.
    - Hatchery tanks from rearing room 3 had apparent decreased odds to be culled.

An association between the following variables and being culled, remained significant ($p<0.05$) using general logistic regression analysis, however, did not remain significant using more complex statistical analysis models ($p>0.05$);

4. Performing in water treatment of larval rearing tanks with Erythromycin (5 mg/L) performed at once, or two or more times.
    - Hatchery tanks that had two or more Erythromycin treatments during larval rearing had an apparent increased odds to be culled.
    - Hatchery tanks that had just one Erythromycin treatments during larval rearing had an apparent had apparent decreased odds to be culled.
5. Tanks stocked with PLs of Northern territory (NT) broodstock genetics.
    - Hatchery tanks stocked with Northern territory (NT) broodstock genetics had increased odds to be culled.
6. Absence of detection of zon occludens toxin gene in routine pre-stocking sampling (~PL 9-11)
    - Detection of zon occludens toxin gene in PL populations during routine pre-stocking sampling had apparent decreased odds to be culled.

Not Assessed Potential Risk Factors

Additional potentially significant risk factors that differed from reported optimal hatchery conditions in peer reviewed literature were identified from the hatchery, however they were not able to be included in the epidemiological analysis due to insufficient available data for analysis. These risk factors included:

1. Elevated total ammonia nitrogen (TAN) and unionised ammonia (NH_3) in larval rearing tanks and live artemia culture water.
2. Elevated salinity in larval rearing tanks and live artemia culture water.
3. Elevated pH in larval rearing tanks and live artemia culture water.
4. Live and artificial feed (algae, artemia, pellet) rearing water concentration and frequency of addition.
5. Feeding of frozen/thawed artemia.
6. Timing of and volume of daily water exchange
7. Chemical disinfectant additions (e.g. Virkon Aquatic).
8. Total viable bacterial counts within larval rearing tanks and tank inputs (e.g. feed).
9. Timing of removal of larval rearing tanks black plastic covering.
10. Probiotic addition (type, frequency, concentration).
11. Acclimatisation (time and magnitude of change) of nauplii into larval rearing tanks.
12. Elevated water temperature

No Association Factors

No association between the following variables and being culled was found ($p>0.05$) using general logistic regression analysis.

1. Hatchery tanks with detected ciliated protozoal parasites during larval rearing.
2. Hatchery tanks with a detected average severity over 1 ciliated protozoal parasite during larval rearing.
3. Hatchery tanks stocked with PLs from East Coast (EC) broodstock genetics
4. Hatchery tanks from rearing room 2
5. Hatchery individual tank

No association between the following variables was found ($p>0.05$) using general linear regression analysis. The regression slope was not different from '0'.

1. Detection of YaFO, or RtX toxin genes in routine pre-stocking sampling (~PL 9-11) and cumulative hatchery survival.
2. Average YaFO, RtX, or zon occludens toxin gene CT value (0-40) and;
    a. Estimated PL population survival at date of sampling.
    b. Timing of previous erythromycin treatment.
    c. Timing of previous Oxytetracycline treatment.
    d. Timing of previous antibiotic treatment.

There was no significant relationship between being culled and any potential pathogens detected in the hatchery analysis.

4.1.2. Grow-out Ponds

Detailed data analysis using STATA v15.1 was performed on 63 different potential risk factor variables within grow out ponds to explore strength of association with being culled.

See full detailed results in Appendix 1 and 3.

High Probability Risk Factors

A strong association between the following variables and the being culled, remained highly significant ($p<0.01$) throughout all analysis and comparisons performed;

1. Decreased time between initial pond filling and stocking PLs into a grow out pond.
    - Stocking grow-out ponds with PLs 11 days or more after completion of pond filling reduced the odds to be culled by 99.9% (highly significant, $p<0.001$), compared to stocking PLs into grow-out ponds less than 11 days after completion of pond filling.
2. Detection of elevated levels of the algae species Nitzschia during monitoring in the post stocking period.
    - The odds of a grow-out pond that had a maximum Nitzschia score of 5 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 61.0 times greater (highly significant, $p<0.001$), than the odds of a grow-out pond that had a maximum Nitzschia score below 5.
3. Reduced pond salinity levels during the post stocking period.
    - Grow-out ponds with a minimum salinity of 36ppt or higher during the first 6 weeks post stocking had reduced odds to be culled by 98.6% (highly significant, $p<0.001$), compared grow-out ponds with a minimum salinity below 36ppt.

Low Probability Risk Factors

An association between the following variables and the being culled, was found to be significant ($p<0.05$) using mixed effects logistic regression analysis. However, no longer remained significant ($p>0.05$) when analysing alongside other significant variables due to correlation or collinearity interactions;

1. Detection of reduced levels of the algae species Cyanobacteria during monitoring in the post stocking period.
    - Grow-out ponds with an average cyanobacteria score of 8 or higher (0=absent; 5=low; 10=medium; 15=high) over the post stocking period (30 days) had apparent reduced odds to be culled.
2. Detection of elevated levels of the algae species Gymnodinium or Nitzschia during monitoring in the post stocking period.
    - Grow-out ponds that had a maximum Gymnodinium score higher than 5 (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking had apparent increased odds to be culled.
    - Grow-out ponds that had an average Gymnodinium score of 1 or higher (0=absent; 5=low; 10=medium; 15=high) or higher over the first 30 days post stocking had apparent increased odds to be culled.
    - Grow-out ponds that had an average Nitzschia score of 2 or higher (0=absent; 5=low; 10=medium; 15=high) or higher over the first 30 days post stocking had apparent increased odds to be culled.
3. Increased fluctuation in water quality parameters (pH, salinity) during the initial the post stocking period.

- Grow-out ponds that had an average daily pH change of 0.15 or higher during the post stocking period (30 days) had apparent increased odds to be culled.
- Grow-out ponds that over the post stocking period (6 weeks) had a maximum salinity change of 6 ppt or higher had apparent increased odds to be culled.

4. Stocking younger PLs into Grow-out ponds.
    - Grow-out ponds stocked with PL of a minimum age of PL10 or older had apparent reduced odds to be culled.
5. Stocking of PLs with lower levels (higher CT value) of zon occludens toxin gene detected in pre-stocking routine sampling.
6. Grow-out ponds stocked with PL that had an average zon occludens toxin gene CT value of 34 or higher, pre-stocking, had apparent increased odds to be culled.
7. Not using chemical piscicide during grow-out pond preparation prior to stocking PLs.
    - Grow-out ponds that used a piscicide during pond preparation had apparent reduced odds to be culled.
8. Decreased time between initial pond fertilisation and stocking PLs into a grow out pond.
    - Grow-out ponds that were stocked 10 days or more after initial fertilisation had apparent reduced odds to be culled.

An association between the following variables and being culled, remained significant ($p<0.05$) using general logistic regression analysis, however, did not remain significant using more complex statistical analysis models ($p>0.05$);

9. Increased fluctuation in water quality parameters (pH, DO) during the initial the post stocking period.
    - Grow-out ponds that during the post stocking period (30 days) had a maximum daily pH change of 0.4 or higher had apparent increased odds to be culled.
    - Grow-out ponds that during the post stocking period (30 days) had a maximum daily DO change of 9mg/L or higher had apparent increased odds to be culled.
10. Increased water quality parameters (pH, temperature, DO) during the initial the post stocking period.
    - Grow-out ponds that during the post stocking period (30 days) had a maximum daily pH of 8.9 or higher had apparent increased odds to be culled.
    - Grow-out ponds that during the post stocking period (30 days) had a maximum daily water temperature of 31°C or higher had apparent increased odds to be culled.
    - Grow-out ponds that had a minimum DO of 8 mg/L or higher during acclimatisation (3 hours post stocking) had apparent increased odds to be culled.
11. Decreased water quality parameters (TAN, DO) during the initial the post stocking period.
    - Grow-out ponds that had an average total ammonia nitrogen (TAN) of 0.5 mg/L or higher over the post stocking period (30 days) had apparent reduced odds to be culled.
    - Grow-out ponds that over the post stocking period (30 days) had a maximum total ammonia nitrogen of 1.5 mg/L or higher had apparent reduced odds to be culled.
    - Grow-out ponds that had a minimum daily DO of 4 mg/L or higher had apparent reduced odds to be culled.
12. Detection of reduced levels of the algae species mixed dinoflagellates during monitoring in the post stocking period.

- Grow-out ponds that had an average mixed dinoflagellate score of 4 or higher (0=absent; 5=low; 10=medium; 15=high) or higher over the first 30 days post stocking had apparent reduced odds to be culled.

13. Smaller Pond Size/volume and/or younger pond ages
    - Grow-out ponds that are smaller (12ML) and/or newer had apparent increased odds to be culled.
14. Stocking younger PLs into Grow-out ponds.
    - Grow-out ponds stocked with PL of a maximum age of PL13 or older had apparent reduced odds to be culled.
15. Stocking ponds with PLs that had multiple Oxytetracycline hydrochloride (20 mg/L) treatments performed in the hatchery.
    - Grow-out ponds stocked with PLs treated with Oxytetracycline 4 or more times during larval rearing had apparent increased odds to be culled.
16. Decreased zon occludens toxin gene (CT value) in routine pre-stocking sampling (~PL 9-11)
    - Grow-out ponds stocked with PL that had an average zon occludens toxin gene CT value of 34 or higher, pre-stocking, had apparent increased odds to be culled.
17. Stocking ponds with PLs that had fewer Erythromycin (5mg/L) treatments performed in the hatchery.
    - Grow-out ponds stocked with PLs treated with two or more Erythromycin during larval rearing had apparent reduced odds to be culled.
18. Stocking grow-out ponds at a lower PL density
    - Grow-out ponds stocked with PL at a density of 70 per sqm or higher had apparent reduced odds to be culled.
19. Stocking grow-out ponds over an extended period of time.
    - Grow-out ponds stocked PLs over an extended period of 9 or more days had apparent increased odds to be culled.

No Association Factors

No association between the following variables and the being culled was found ($p>0.05$) using general logistic regression analysis.

1. Stocking grow-out ponds with PLs that had one or more Oxytetracycline (20mg/L) or Erythromycin (5mg/L) treatments in the hatchery.
2. Grow-out ponds with an increased average algae species monitoring score.
    - Average *Cyanobacteria* spp, or maximum *Cyanobacteria* spp. score, or maximum mixed dinoflagellates
3. Water quality parameters (pH, temperature, DO, salinity) during the initial the post stocking period (30 days).
    - average daily DO change, or average daily DO, or maximum daily DO
    - average daily pH, or maximum daily pH,
    - average water temperature, minimum daily water temperature, maximum water temperature, or average daily water temperature change, or maximum daily water temperature change,
    - maximum salinity.

4. Stocking grow-out ponds with PLs that had low or high survival in the throughout larval rearing.
5. Stocking grow-out ponds with PLs above or below PL11.
6. Average health score or stress test score prior to stocking grow-out ponds.
7. YaFO, or RtX, or zon occludens toxin genes in routine pre-stocking PL sampling (~PL 9-11)
    - Average CT value (RtX and YaFO only), or minimum CT value, or Presence (positive detection).

There was no significant relationship between the case definition (0% survival) and any potential pathogen detected in the grow-out analysis.

4.1.3. Prospective Trial Grow-out Ponds

Comparison to the case definition

All three of the prospective trial ponds were maintained to harvest with adjusted post-harvest estimations on pond survival of

- Pond 43: 22.83% (2065 Kg)
- Pond 44: 97.80% (7572 Kg)
- Pond 45: 29.32% (2655 Kg).

Therefore, all three of the trial ponds did not meet the case definition (culled pond, 0% survival).

Comparison to commercial grow-out ponds

A comparison between the data from prospective trial ponds and the commercial grow-out pond epidemiology analysis findings (see above) was performed. The trial ponds were found to align with the with non-case (>0% survival) populations for the following potential risk factors:

High Probability Risk Factors

- Maximum levels of the algae species *Nitzschia* during monitoring in the post stocking (30 day) period (pond 45 only).

Low Probability Risk Factors

- Average levels of the algae *Nitzschia* species during monitoring in the post stocking (30 day) period (pond 45 only).
- Maximum salinity change during the post stocking period (6 weeks) (all three trial ponds).
- Minimum PL age at stocking (all three trial ponds).
- Maximum PL age at stocking (all three trial ponds).
- Minimum DO during post stocking acclimatisation (3 hours) (all three trial ponds).
- Maximum daily water temperature during the post stocking (30 days) period (Pond 43 only)
- Minimum daily DO during post stocking period (30 days) (Pond 43 only).
- Number of days used to stock PLs into a grow-out pond (all three trial ponds).

All additional potential risk factors not listed above were found to align with the case (>0% survival) in the three trial ponds.

Comparison to within trial grow-out ponds

A comparison of potential risk factors between the three prospective trial pond data was performed to assess differences between higher (Pond 44, n=1) and lower (Pond 43 and 45, n=2) harvest survival trial ponds (See also Appendix 3).

During the post stocking period (30 days), the higher survival pond (pond 44) was found to have;

- Lower maximum daily pH, average daily water temperature change, maximum daily water temperature change, minimum daily DO, and average *Gymnodinium* sp. Score.
- Higher - maximum daily DO change, maximum total ammonia nitrogen (TAN), minimum salinity (ppt), average *Cyanobacteria* sp. score and average mixed dinoflagellates score.
- Lower average YaFO, RtX CT value in the pre-stocking PL qPCR testing (day 0 sample).

4.2. Development of a qPCR for bacterial toxin genes identified in Next Generation Sequencing (NGS) of bacterial cultures collected from APF water during the 2018 hatchery season

4.2.1. Next Generation Sequencing (NGS)

NGS performed on mixed bacterial isolates of homogenised whole PL and culture tank water from hatchery tanks with elevated mortality (Run 3; Tanks 27, 30, 36 and 38; collected on 15/10/18) identified three toxin genes (zon occludens, RTX and YaFO) in high relative abundance. These toxin genes were identified as suitable targets for further investigation as potential contributing factors in the hatchery (and grow-out pond) mortalities.

4.2.2. Development of the toxin gene qPCR

A qPCR diagnostic test was developed for toxin genes: zon occludens, RTX and YaFO. This qPCR was subsequently performed on 135 PL population samples collected from the APF hatchery during the 2018 season and 1 timepoint of post stocking PL populations (day 0) from the prospective trial ponds (see sections below).

4.2.3. Repeatability of the toxin gene qPCR

The detection of each of the three toxin genes was highly consistent (See Table 6) and highly repeatable (>95%, See Table 5) between the five individually extracted replicates from each PL population sample tested.

There was perfect agreement in the presence or absence of the toxin gene testing output in over 95% of testing PL batch populations (Table 5).

Table 4. Assessment of the consistency of toxin gene detection (presence/absence) within all tested hatchery PL population samples tested during the project from run 1, 3, and 4.

Toxin gene target:	***YaFO***	***RtX***	***Zon occludens***	***Total***
Sample count	100	100	135	335
Perfect agreement count (all 5 replicates yield the same output)	96	96	129	321
Perfect agreement percentage (all 5 replicates yield the same output)	96	96	95.6	95.8

The use of 5 replicates from within a homogenised PL batch sample pool was not found to result in a substantially different qPCR CT value output, when compared to testing only 2 replicates (Table 6).

Table 5. Assessment of the consistency of toxin gene quantification (CT value repeatability coefficient) within all tested hatchery PL population samples tested during the project from run 1, 3, and 4.

	Average Repeatability Coefficient (Minimum-Maximum)		
Toxin gene target:	**YaFO**	**RtX**	**Zon occludens**
All 5 sample replicates (n=1 possible combination)	3.0641	2.7918	2.8580
4 sample replicates (n=5 possible combinations)	3.0602 (2.8151-3.1675)	2.7829 (2.5200-2.8955)	2.8565 (2.7485-2.9876)
3 sample replicates (n=10 possible combinations)	3.0543 (2.7184-3.3263)	2.7667 (2.4373-3.0909)	2.8528 (2.7099-3.1259)
2 sample replicates (n=10 possible combinations)	3.0395 (2.5069-3.6002)	2.7403 (2.3636-3.3375)	2.8433 (2.3894-3.3085)

4.3. The presence of putative bacterial toxins in PL samples from APF larval tanks experiencing elevated mortality during the 2018 hatchery season

4.3.1. Toxin genes (YaFO, RtX, and zon occludens)

Toxin gene qPCR testing performed on six hatchery PL populations (Run 3 – Tanks 26, 27, 30, 36, 37 and 38; n=6), experiencing elevated mortality at the time of sampling, detected all three toxin genes in 100% (6/6) of the samples tested. There was an apparent increase in the load of all three toxin genes (lower CT value), relative to routine samples collected from PL population that did not have elevated mortality detected (See Table 7).

The toxin genes RtX and zon occludens were first detected in run 1 from 2/17 routine hatchery PL populations samples (tanks that subsequently suffered elevated mortality, case tanks (0% survival)) and 1/52 routine hatchery PL populations samples (both case and non-case tanks), respectively. No samples from run 2 were available for testing. The toxin genes YaFO, RtX and zon occludens were detected in run 3 and 4 from all routine hatchery PL population samples (both case and non-case tanks).

Table 6. Comparison of the zon occludens, RTX and YaFO toxin gene average CT values from 'opportunistic' PL samples collected from hatchery tanks (Run 3; Rearing Room 2; Tanks 26, 27, 30, 36, 37 and 38; PL3-6) following the detection of elevated mortality (PL3-6), and 'routine' PL samples collected from hatchery tanks (Run 3; Rearing Room 3; Tanks 45-48, 50, 51, 53-67; PL9-12) prior to stocking and that were apparently healthy.

	Toxin gene CT value		
Toxin gene target:	**YaFO**	**RtX**	**Zon occludens**
'Opportunistic' hatchery PL samples collected from apparently unhealthy populations during a mortality event (n=6)	21.75 ± 0.32	22.85 ± 0.25	24.92 ± 0.32
'Routine' hatchery PL samples collected from apparently healthy populations prior to stocking (n=21)	31.19 ± 0.61	31.42 ± 0.57	33.69 ± 0.56

Comparison between hatcheries

Some very limited testing of historic PL population samples from a few different hatcheries, including APF, detected these YaFO, RtX and zon occludens toxin genes in some other hatcheries in the industry, not just APF (data not available).

4.4. Potential pathogens involved in elevated mortality experienced in the hatchery and grow-out production stages at during the APF 2018/19 season

4.4.1. Hatchery

The APF hatchery experienced unprecedented rapid onset tank-based mortality events between July and November 2018. The hatchery production had an estimated overall season survival of 12.3% (120/260 tank PL populations, approx. 29.2 million PL) and a non-survival of 88.7% (140/260 tank PL populations, approx. 208.5 million PL).

Parasitology

In September 2018, ciliate parasites were detected in several hatchery larval tanks that were experiencing mortality. However, these parasites were equally detected in several hatchery tanks not experiencing mortality. Further identification advice from the University of Queensland suggested the ciliate parasite to be identified as hypotrich ciliates (Such as *Oxytricha* sp. or *Euplotes* sp.). Histopathology performed on PL populations (Run 2 – Tanks 3, 21; Run 2 Tanks 45, 49) experiencing elevated mortality at the time of sampling, tested at BSL, did not detect ciliate parasites in any of the submitted samples.

Virology

In Early-mid 2018, broodstock screening testing performed at JCU did not detect any viral pathogens of concern that would trigger further action.

In September 2018, samples collected from PL populations (Run 2 – Tanks 3, 21; Run 2 Tanks 45, 49) experiencing elevated mortality at the time of sampling, were tested at BSL for pathogens of concern (Ref -P18-04857). YHV-7 and MoV were not detected in any of the four hatchery tank PL samples. IHHNV were detected in 50% (Tank 3 and 45) and GAV 25% (Tank 3) hatchery tank PL samples. There was no histopathological evidence for viral induced pathology detected.

In January 2019, samples from PL populations (Run 3 – Tanks 26, 27, 30, 36, 37 and 38; n=6), experiencing elevated mortality at the time of sampling, were tested at AAHL for pathogens of concern (See appendix 2). CMNV, IMV, LsV, MoV, SHIV, TSV, WSSV, YHV-1, YHV-7 were not detected in any of the samples. GAV was detected in 33% (2/6) of the samples. The pattern of detections did not suggest that GAV was the causative agent of the hatchery losses.

Bacteriology and Mycology

In September 2018, a sterile saline flushed tank scraping sample from a single hatchery tank that had just been culled due to rapid onset mortality was streaked onto TSA and TCBS agar plates and sent to BSL to be cultured (Ref - P18-04904). Major finding included: *Vibrio* spp., *Vibrio alginolyticus, Bacillus* sp., *Micrococcus* sp., were isolated in mixed growth. Histopathology detected proliferating bacterial on organs and tissues of dead PLs, bacteria proliferation of mid-gut (MG) and hepatopancreas (HP) with necrosis of mucosa with rounding and sloughing of cells, and melanisation of shell cuticle was detected (consistent in all tank samples submitted). No fungi were consistently detected on PL samples from affected tanks.

In January 2019, samples from PL populations (Run 3 – Tanks 26, 27, 30, 36, 37 and 38), experiencing elevated mortality at the time of sampling, were tested at AAHL for pathogens of concern (See appendix 2). AHPND VpPirA, EHP and NHP were not detected in any of the samples.

No aseptically collected samples of homogenised apparently healthy, dead or dying PL were collected and submitted for bacteriology testing during the mortality investigation.

4.4.2. Grow-out

The APF grow-out farm experienced unprecedented pond-based mortality during the early crop (post stocking period, 6-8 weeks) with prawns failing to materialise on feed trays as detected from December 2018. Grow-out production from the initial pond stocking, had an estimated average survival of 11%, producing approximately 3.6 million prawns harvested from 20 of the 47 initially stocked ponds.

Parasitology

In February 2019, samples collected from pond number 27 which was experiencing elevated morbidity near harvest, were tested at BSL for pathogens of concern (non-case pond, 23% adjusted harvest survival; Ref -P19-00811). Extensive cuticle fouling with sessile ciliates and granular debris was found in 2/3 prawns examined. No ectoparasites were detected on prawn samples collected from any other affected ponds.

The cause of low survival in these ponds was not apparent from the examination of the sampled prawns.

Virology

In December 2018, approximately 6 weeks post stocking, samples collected from pond populations (Pond 4, 6, 45, 7, 14 and 5), that had experienced elevated mortality in the post stocking period, were tested at BSL for pathogens of concern (See appendix 3; Ref- P18-06072F, P18-06074F, P18-06077F, P18-06075F, P18-06076F, P18-06073F, P18-05992F). TSV, WSSV, YHV-1, YHV-7 were not detected in any of the samples. IHHNV (suspect positive) was detected in 8% (4/52), and GAV was detected in 62% (27/52) of samples. Histopathology detected increased spheroids in 8% of prawns (2/5 prawns).

In January 2019, approximately 8 weeks post stocking, samples collected from pond 5 during a drain harvest, of another very low survival pond, were tested at BSL for pathogens of concern (See appendix 3; Ref- P19-00170). Histopathology detected increased spheroids in 67% of prawns (4/6), and central muscle fibrosis in 67% of prawns (2/3).

In February 2019, samples collected from pond 27 (see details above) were tested at BSL for pathogens of concern. TSV, WSSV, YHV-1, YHV-7, were not detected in any of the samples. IHHNV and GAV were detected in 100% (3/3) of samples.

The cause of low survival in these ponds was not apparent from the examination of the sampled prawns.

Bacteriology and Mycology

In December 2018, samples collected from pond populations ((Pond 4, 6, 45, 7, 14 and 5) that had experienced elevated mortality in the early post stocking period, were tested at BSL for pathogens of concern. PirA/Pir B were not detected in any of the samples. Histopathology detected marked necrosis and inflammation of the hepatopancreas with necrotic ducts and gram-negative bacteria in 4% of prawns (1/25), an attached moult shell was in 16% of prawns (4/25), haemocyte infiltration of interstitial tissue in 4% of prawns (1/25), and no significant abnormalities in 96% of prawns (24/25).

In January 2019, samples collected from pond 5 (see above) were tested at BSL for pathogens of concern. PirA/Pir B were not detected in any of the samples. Aseptic hepatopancreas and haemolymph samples were streaked onto TCBS and TSA agar plates and cultured. A light pure to moderate mixed growth of the *Vibrio harveyi* (50% or 3/6 prawns), *Vibrio parahaemolyticus* (50% or 3/6 prawns), *Photobacterium damselae* (17% or 1/6 prawns) were isolated. Histopathology detected severe haemocytic enteritis (HE) in the entire mid-gut 50% of prawns (3/6) and an attached moult shell in 50% of prawns (3/6).

In February 2019, samples collected from pond 27 (see details above) were tested at BSL for pathogens of concern. PirA/Pir B were not detected in any of the samples. Histopathology detected thin, sloughing, melanised and bacteria lined hepatopancreatic tubules in 100% of prawns (3/3), haemocytic enteritis (HE) in 33% of prawns (1/3), an attached moult shell in 67% of prawns (2/3), and no to low intracytoplasmic vacuolation in 100% of prawns (3/3).

No fungi were consistently detected on any submitted specimens.

The consistent cause across all ponds was not apparent from the examination of the sampled prawns.

4.5. Prospective trial pond survival and performance

Day to day management of the prospective grow-out pond trial was performed by APF staff (Skye Tara Lewis, Matt West and Andrew Smith) with oversight from FFVS. In addition, FFVS assisted initial stocking and commencement of the trial, and a trial mid-point assessment. JCU Aqua path lab provided technical assistance to farm staff regarding molecular biology aspects throughout the trial.

4.5.1. Trial Commencement

On the morning of 10/01/19, approximately 1.2 million PL20 sourced from PRF hatchery tanks N4 and N3 were harvested into three tankers (~400,000 PL per transporter) for transport to the APF Ilbilbie grow-out pond farm.

Water quality parameters (Temperature, DO, pH, salinity) were monitored pre-harvest (Table 8), prior to and during transport (Table 9), and on arrival APF during acclimatisation (3 hours post stocking, data not shown).

Transporter tanks were acclimatised with destination pond water for approximately 1-2 hours prior to release (stocking) into each pond at near 3pm on 10/01/19.

Upon stocking all three trial PL populations passed the freshwater stress test (100% survival) and initial health assessment (Figure 1), with only a significant size variation (in all three pond populations) detected.

Table 7. Water Quality measurements from Pacific Reef Fisheries (PRF) hatchery tanks containing PLs destined for the APF prospective trial ponds (43, 44, 45).

Tank ID	*Salinity (ppt)*	*Water Temperature (°C)*	*pH*	*Dissolved Oxygen (DO, mg/L)*
N3	38.1	28.1	7.84	6.14
N4	37.9	28.5	7.45	7.67

Table 8. Water Quality measurements from APF transporter tanks prior to transport containing PLs destined for the prospective trial ponds (43, 44, 45).

Transporter ID	*Target Pond*	*Salinity (ppt)*	*Water Temperature (°C)*	*pH*	*Dissolved Oxygen (DO, mg/L)*
1	43	37.0	28.5	8.01	12.1
2	44	36.7	28.7	8.19	13.5
3	45	36.7	28.8	8.10	15.2

Figure 1. Truck loaded with transport tankers used for transfer of PLs from hatchery to prospective trial ponds

4.5.2. Trial Progression

Diagnostic samples for field health assessment, histopathology and molecular biology were successfully collected from all three trial ponds, 3 times per week, throughout the entire 56 day trial (See Section 4.6.).

The only detected mortality was in pond 43, with two dead PLs detected on a feed tray five days after commencing the trial (14/01/19). No further dead PLs were detected on the following health check two days later (16/01/19), or at any later time point in pond 43 or at any stage in pond 44 and 45. None were observed on pond edges or on trays at any other timepoint.

Average health score improved over time for each pond for the duration of the trial (See Figure 1). The large size variation (as detected upon stocking) remained present throughout the trial (estimated at < 70% of PL at the same size at stocking). Size variation (coefficient of variation) at day 53 of the trial (04/03/19) within each of the three trial ponds was 60%, 54% and 44% for pond 43, 44 and 45, respectively (See Table 10). Average liveweight (g) and length (mm) steadily increased in all three trial ponds (Figure 2 and 3) over the duration of the trial (See Appendix 1).

Daily water quality monitoring (ex. NH_3 and salinity which was monitored weekly) data can be found in Table 11. Except for NH_3, there was no large differences between the three trial ponds. Pond 44 had the highest TAN and NH_3 (near 0.3 mg/L) recorded approximately 7 weeks post stocking.

4.5.3. Trial Outcome

The trial was ended on 07/03/19 (56 days after commencement). No case ponds (0% survival) occurred. Survival to harvest was estimated for each of the three trial ponds at this timepoint as per normal APF protocol. At 8 weeks post stocking estimations on pond survival to harvest was: Pond 43 = 35%, Pond 44 = 80%, Pond 45 = 35%. The prospective trial ponds where taken through to harvest with the following adjusted post-harvest estimations on pond survival of: Pond 43 = 22.83% (2065 Kg), Pond 44 = 97.80% (7572 Kg), Pond 45=29.32% (2655 Kg). Pond 43 and 45 were below the historic anticipated farm average, with pond 44 above the historic farm average.

Table 9. Comparison of the individual prawn average, minimum and maximum liveweight (g) and standard deviation (SD) and coefficient of variation (CV, %) within trial pond 43, 44, and 45 at day 53 (4/3/19) of the prospective grow-out trial.

Pond ID	*Average Weight (g)*	*Minimum Weight (g)*	*Maximum Weight (g)*	*S.D.*	*C.V. (%)*
43	5.38	1.45	15.89	3.20	59.5
44	2.96	1.09	7.68	1.60	54.2
45	3.41	0.87	8.22	1.49	43.8

Table 10. Water Quality measurements from APF grow-out trial ponds 43, 44, and 45 over the duration of the 56-day trial.

Pond ID		*Salinity (ppt)*	*pH*	*Water temperature (C)*	*Dissolved oxygen - DO (mg/L)*	*Unionised ammonia - NH_3 (mg/L)*
43	Av.	30.3	8.58	28.1	8.07	0.01
	Max	32.5	9.14	31.4	15.57	0.06
	Min	27.6	7.87	24.5	3.80	0.00
44	Av.	30.7	8.51	28.1	7.39	0.03
	Max	32.8	9.18	30.5	14.10	0.29
	Min	28.0	7.65	24.5	3.00	0.00
45	Av.	30.7	8.79	28.1	7.85	0.01
	Max	32.5	9.23	30.3	14.90	0.05
	Min	28.1	8.37	24.6	3.40	0.00

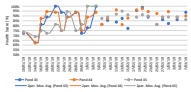

Figure 2. Comparison the prawn health score (Swimming Activity, Stress Test, Size variation, Morphology/ Deformity, Fouling, Intestine, Muscle: Gut Ratio, Hepatopancreas, Gills) in trial ponds (43-45) for duration of the trial. See methods for more details. Moving average shown by solid line.

4.5.4. Molecular Biology

Pre-stocking (day 0, 10/01/19) homogenised whole PL samples from all three trial pond populations were tested via qPCR for all three target toxin genes at JCU Aquapath lab. RtX, YaFO, and zon occludens, toxin genes were detected in all three samples tested. CT values for pond 43, 44 and 45 were not available to the time of writing the report.

No further molecular testing of prospective trial molecular samples was commenced after consideration of steering committee.

4.5.5. Histopathology

To identify pathology changes associated with apparent survival differences in trial ponds samples were taken on day 0, 4, 6, 8, 11, 13, and 15 post stocking from each of the three trial ponds and histological analysis was performed by BSL.

The majority of the prawns were processed as entire animals with microtome trimming through the tissues so that a mid-sagittal section of each animal was apparent histologically. It was not practically possible to ensure every animal was in the same horizontal plane in the tissue block, so not every organ was apparent in every prawn examined histologically. Samples from day 13 and 15 from each trial pond were dissected mid-sagittal through the thorax and transversely through a half thorax and the abdomen prior to processing into blocks – this helped to ensure most major organs were present in sections. In all prawns the hepatopancreas was present; most had, at least part, of the midgut in section; about 60% had foregut in section; and in about 30% of the prawns a little gill tissue was be examined.

Table 11. Number of prawns examined in histological sections at each time point post stocking into grow-out trial ponds (43, 44 and 45)

Pond ID	*Day 0*	*Day 4*	*Day 6*	*Day 8*	*Day 11*	*Day 13*	*Day 15*	*Total*
43	33	32	32	19	34	42	37	229
44	43	22	19	35	19	38	29	205
45	31	17	12	22	35	34	34	185

Details of pathology observed in each pond can be found in Table 13.

Size variation appeared to increase as the prawns got older within all three trial ponds. Unanticipated autolysis was apparent in majority of the preserved samples except for the smaller individuals, which restricted histological interpretation. Lymphoid tissue appeared to increase over time in all ponds (Pond 43: 27% (d0) to 46% (d15); Pond 44: 12% (d0) to 59% (d15); Pond 45: 29% (d0) to 47% (d15).

Additional histological analysis was commenced on pond 43 samples (the pond that experienced the lowest survival) for samples between day 25 and 35 (timepoints day 25, 27, 29, 32, 34) to identify pathology changes associated with apparent survival differences. During preliminary histological examination of a sub-sample (d32 and d34) it was found that 5-100% of the hepatopancreas of these samples was autolytic with loss of hepatopancreas tubular architecture. Further examination of these samples was not performed due to inadequate tissue preservation impairing the opportunity for accurate interpretation.

Table 12. The occurrence of histological findings (and apparent prevalence during the period) in examined prawns (43, 44 and 45) during the first two weeks post stocking into grow-out trial ponds.

	Pond 43 (n=229)		***Pond 44 (n=205)***		***Pond 45 (n=185)***		***Total (n=619)***	
Histological Finding	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Attached moult shell	4	1.7	4	2.0	10	5.4	18	2.9
Proliferation of bacteria (mandible)	19	8.3	19	9.3	4	2.2	42	6.8
Proliferation of bacteria (oesophagus)	0	0.0	0	0.0	1	0.5	1	0.2
proliferating bacteria (hindgut)	0	0.0	0	0.0	1	0.5	1	0.2
Granuloma (mandible)	4	1.7	1	0.5	0	0.0	5	0.8
Melanisation (gill)	1	0.4	2	1.0	1	0.5	4	0.6
Melanisation (mandible)	5	2.2	6	2.9	2	1.1	13	2.1
Melanisation (pleopod)	4	1.7	0	0	2	1.1	6	1.0
Melanisation (abdominal cuticle)	0	0.0	0	0.0	1	0.5	1	0.2
Haemocyte infiltration (hepatopancreas)	0	0.0	0	0.0	1	0.5	1	0.2
Spheroid formation (within lymphoid tissue).	11	4.8	15	7.3	22	11.9	48	7.8
Spheroid formation (thoracic / abdominal connective tissue).	2	0.9	10	4.9	19	10.3	31	5.0
Spheroid formation (heart)	0	0.0	1	0.5	2	1.1	3	0.5
Spheroid formation (antennal gland)	0	0.0	1	0.5	0	0.0	1	0.2
Abdominal muscle necrosis	1	0.4	0	0.0	0	0.0	1	0.2
Fouling of the cuticle with sessile peritrichous ciliates.	0	0.0	24	11.7	0	0.0	24	3.9
Presence of lymphoid tissue	69	30.1	54	26.3	67	36.2	190	30.7

4.6. Post-stocking sampling methods for PL's to monitor health status

Regular and reliable capture of post stocked, free ranging post larvae was achieved in all three of the prospective trial ponds (Ponds 43, 44, 45) using feed trays (see Figure 3). Standard prawn feed trays with course black mesh were used successfully for obtaining post stocking prawns samples. This activity was performed 3 times per week, throughout the entire duration of the trial, with a total of 23 sample time points, for each pond, collected for diagnostics.

It was observed that the number of PLs retrieved from each tray would appear to decrease if the trays were not kept clean from fouling (e.g. every 2-3 days). Capture of enough PLs for sampling was achieved through maintaining at minimum 3 feed trays in each pond at all times of the trial. The PLs appeared in highest numbers in feed trays near the point of release, initially, then were found on feed trays around the pond (data not available).

The average relative abundance of prawns observed on feed trays appeared to weakly correlate with differences in end harvest survival between each of the trial ponds. From day 25 (3.5weeks) of the prospective trial, onwards, the average number of PLs found on pond 44 feed trays was higher (Av. 55 PLs) than pond 45 (Av. 26 PLs), pond 43 (Av. 15 PLs), and the three pond average (Av. 32 PLs) (Figure 2). This order reflected that of end harvest survival.

Figure 3. APF farm staff (Skye) retrieving feed trays containing numerous prawns from prospective trial ponds for diagnostic sampling during the trial.

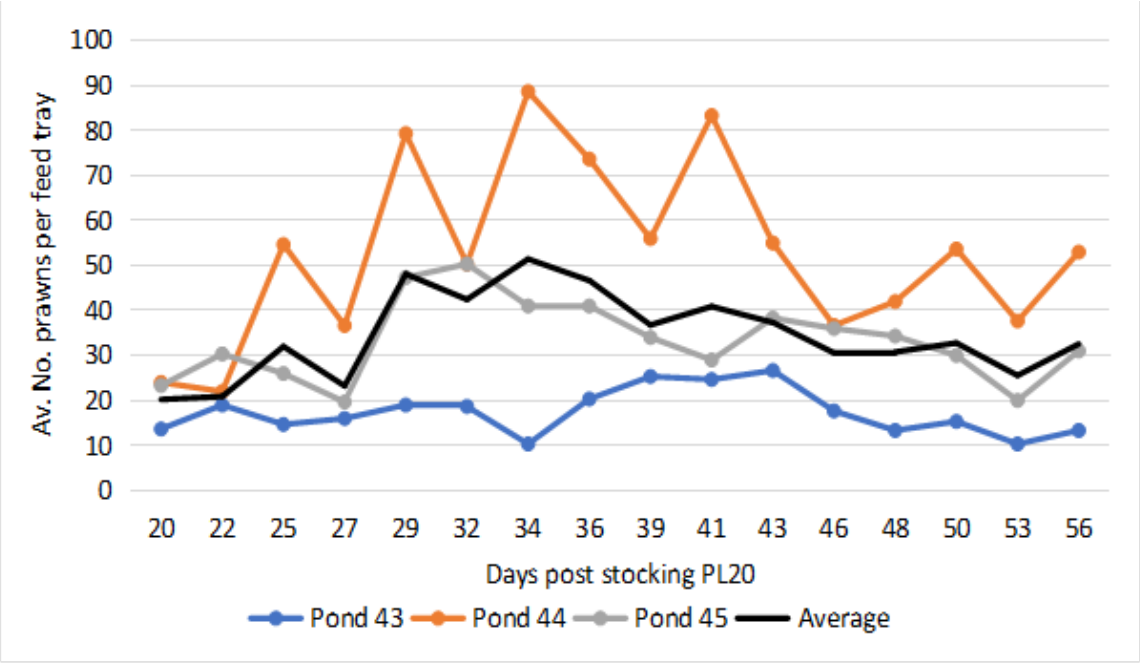

Figure 4. Comparison of the average prawn numbers found on each feed tray (n=3) at each sampling time point, following stocking of PL20 in trial pond 43-45 and the combined average of these three ponds, for the duration of the trial. No data before day 20.

4.7. Levels of agri-chemical pollutants in source water entering ponds and potential impact on prawn larval health

Replicate Empore™ disk passive water samplers deployed into trial pond 44 were calibrated to detect 81 compounds (including personal care products and neonicotinoid contaminants) in the pond water (See Appendix 1). The first batch of samplers were deployed into pond 44 on 14/01/19 (day 4 of the trial) and replaced on 7/2/19 (day 28 of the trial) with the second batch of samplers that remained in pond 44 until the 7/3/19 (day 56, last day of the trial). Passive samplers were analysed by Queensland Alliance for Environmental Health Sciences (QAEHS), Brisbane.

Neonicotinoid insecticides were not detected (See appendix 1).

Time weighted average of detected anthropogenic contaminants identified by the samplers can be found in Table 14 (and Appendix 1).

Table 13. Summary of the passive water sampler pollutant detections in prospective trial pond 44 for the duration of the trial (56 days).

Sample Name	*Average Result*	*Min Result*	*Max Result*
2,4-Dichlorophenoxyacetic acid (2,4 D);	0.48 ng/L	0.07 ng/L	1.02 ng/L
Atrazine	0.51 ng/L	0.20 ng/L	1.05 ng/L
Bromoxynil	0.04 ng	0.04 ng	0.05 ng
ametryn hydroxy	1.20 ng	1.09 ng	1.60 ng
desethylatrazine	0.13 ng	0.09 ng	0.18 ng
diuron	0.69 ng/L	0.35 ng/L	1.21 ng/L
hexazinone	0.56 ng/L	0.26 ng/L	0.75 ng/L
MCPA	0.28 ng/L	0.08 ng/L	0.51 ng/L
Tebuthiuron	0.03 ng/L	0.02 ng/L	0.04 ng/L

5. Discussion

This research project was conducted to identify possible risk factors and/or pathogens associated with or involved in the unprecedented mortality experienced during the 2018/19 APF hatchery and grow-out production. The project sought to develop diagnostics tests and techniques to help identify pathogens, and support improved investigations of future disease events, and guide development of evidence-based prevention and control strategies to assist management of future prawn mortality events in the Australian prawn farming industry.

Limitations and shortcomings were identified and discussed throughout to provide necessary context and caution on the conclusions drawn. This commentary is useful to provide justification and direction to future work recommended by the project.

5.1. Epidemiological analysis of risk factors associated with low survival in the APF 2018/19 commercial hatchery, commercial grow-out pond, and trial grow-out pond populations.

The epidemiological analysis assessed the relationship between potential risk factors (or potential contributory causes) and the case definition (0% survival) using the available commercial farm data sets. This in turn allows for a set of evidence-based recommendations to be implemented to reduce the likelihood of re-occurrence of the case definition (0% survival). This is a common and proven approach when investigating disease in an animal population.

Within a prawn hatchery setting, a similar approach was performed by Kumar (2017), when investigating causes of zoea-2 syndrome in *Litopenaeus vannamei* of Indian hatcheries. In that instance, molecular testing (PCR) of known DNA and RNA virus pathogens did not identify any infectious pathogens that could explain the mortalities experienced (Kumar, 2017). These researchers did find a range of potential risk factors linked to the disease outbreaks and generated a range of recommendations to reduce disease occurrence, including (but not limited to): shortening the nauplii stocking period (length of days) in the same larval rearing unit, improving disinfection between the production cycles, creating further separation and improving biosecurity between the maturation unit, larval rearing unit, and algal culture unit, with separate workers and separate implements for different units. Using microscopy, the researchers also found affected animals appeared to have had reduced feed intake and pathological changes to the hepatopancreas and intestine suggestive of impairment nutrient absorption and starvation in the affected animals, which may have been contributing to the diminishing activity, delayed moulting and mortality. Kumar (2017) concluded that the mortalities were likely due to accrued conditions in the larval rearing tanks during the larval cycles even with constant and uniform water quality parameters and management practices.

Within a grow-out pond setting, a similar approach was performed by Zorriehzahra (2015) (albeit from a position of observation rather than data analysis), when investigating causes of early mortality syndrome (EMS) outbreaks of Sri-Lankan prawn farms. Researchers were seeking to identify potential risk factors to shape disease control and prevention measures. These researchers found a range of potential risk factors linked to the disease outbreaks and generated a range of recommendations to reduce disease occurrence, including (but not limited to): changes to pond preparation (washing, spraying lime, ploughing), reducing pond pH or pH swings, the use of probiotics, stocking healthy and high quality PLs, reducing stocking density, monitoring of water and soil for bacteria (*Vibrio* sp.), use of high quality feed, improving water quality and feeding management to reduce environmental stress, use of large water storage ponds to facilitate maintaining stable water salinity, increasing pond aeration to prevent periods of low dissolved oxygen, and the use of polyculture (shrimp and filter feeding marine fish).

Many of the above highlighted risk factors align with potential risk factors found in this project that were associated with the case definition (0% survival). These are discussed in more detail below.

5.1.1. Hatchery

Three risk factors were found to be significantly associated with the case definition (0% survival) throughout all statistical analysis and comparisons performed on the hatchery data set. These included: merging of hatchery tanks; stocking larval rearing tanks with 900,000 or more nauplii (8000L tanks, equivalent to 112.5 PL per litre); and not treating larval rearing tanks with an Oxytetracycline hydrochloride (20 mg/L) treatment course (3 days) at least once.

Hatchery Tank Merging

The merging of hatchery tanks was performed following detection of mild-moderate mortality in multiple larval rearing tanks to a point where the surviving population were less than half the target stocking density and mortality subsequently appeared to decrease or cease. These tanks were merged or combined into a single population to more economically manage the hatchery tank space and labour resources within the hatchery. This practice did not prove to be effective at yielding surviving harvestable PLs for pond stocking with only 5/43 (12%) of hatchery tanks that had this performed surviving through larval rearing. It is likely that factors contributing to mortality may remain in the previously affected populations, and the additional stressor of merging multiple population, may exacerbate the risk of further mortality.

No literature could be found on the use of merging of larval rearing tanks following detection of mortality as an effective practice. The analysis performed in this project suggested that merging of hatchery tanks should be avoided to increase the probability of survival through larval rearing.

Nauplii Stocking Density

The stocking larval rearing tanks with 900,000 or more nauplii (8000L tanks, equivalent to 112.5 PL per litre) is a standard protocol at APF. Numerous trials have been performed at APF in the past to provide confidence that stocking larvae rearing tanks at approximately 1 million nauplii (125 PL per L) to not have a significant negative impact on survival (data not available). This may be the case in the scenario when those hatchery trials were performed, however, this did not appear to be the case in the 2018 hatchery production season with only 31% (65/200) of hatchery tanks with this stocking density surviving through larval rearing. Commercial production systems are a highly dynamic and ever-changing landscape so many contributing factors, some of which are not fully clear, could explain this change in apparent risk.

There was a strong run effect (temporal effect) on stocking density linked to stocking sequence and therefore time, with 89% (48/54) hatchery tanks stocked at a density less than 900,000, being from run 4. A temporal influence on the mortality pattern is referring to the strong relationship between mortality and time. In order to account for this strong effect, we also analysed data within time sections (e.g. run 1, run 2, run 4) independently from each other to see if when removing this influence, whether the exposure (potential risk factor) remained significant or not. Despite this temporal effect the relationship between elevated stocking density and the case definition (0% survival) remained significant.

The nauplii stocking density targeted at APF is at or above the higher end of some target reference ranges available in the literature, suggesting targeted stocking density should be between 75 and 120 nauplii per litre, assuming a full larval–rearing tank (FAO Fisheries and Aquaculture Department, 2007). The analysis performed in this project suggested that if larval rearing tanks were stocked with less than 900,000 nauplii it could assist in increasing the probability of a tank surviving through larval rearing.

Antibiotic Treatment

The use of oxytetracycline hydrochloride (OTC, 20mg/L) as an immersion treatment course (3 days) via off-label veterinary prescription commenced as a clinical trial in late hatchery run 3. This followed ongoing mortality in early hatchery run 3, despite changes to some aspects of larval nutrition, water quality and husbandry. A bacterial component to the mortality events was identified in histopathology with affected larvae succumbing to enteric bacterial infections, suggestive of Vibriosis. *Vibrio* sp.

were also cultured from an affected hatchery tank microbiological sample. Treatment was aligned with improvements to survival apparent in most of hatchery tanks receiving at least one 3-day treatment course. Only 4% (3/83) of hatchery tanks that received OTC treatment suffered elevated mortality at a level which met the case definition (0% survival).

There was a strong run effect (temporal effect) on oxytetracycline hydrochloride treatment usage, linked to stocking sequence and therefore time, with 100% (59/59) hatchery tanks from run 4, and 0% (0/136) of hatchery tanks from run 1 and 2, receiving OTC treatment. However, despite this effect the relationship between not performing OTC treatment and the case definition (0% survival) remained significant.

The use of Oxytetracycline (OTC) treatment to treat vibriosis appears appropriate given the general efficacy of tetracyclines against gram-negative bacteria (e.g. *Vibrio* spp.). While resistance of *Vibrio* species (6.5%) isolated from in Australian aquaculture sources to OTC has been reported (Akinbowale, Peng, & Barton, 2006), the few Vibrio sp. isolates which were collected from the APF hatchery tank samples were found to have sensitive to intermediate OTC resistance profiles OTC (P18-04904).

Oxytetracycline is a bacteriostatic antibiotic that inhibits proteins synthesis of a wide range of gram positive and negative bacterial species (Teves-Brown, 2000). This antibacterial agent has been used in prawn hatcheries to assist management of some diseases involving bacteria (Sathish Kumar, Vidya, Kumar, Alavandi, & Vijayan, 2017; Uddin & Kader, 2006). Immersion concentrations of oxytetracycline between 5 and 120 mg/L have been suggested, varying with the hardness of the water (Teves-Brown, 2000). However, it is noted that in saltwater a large proportion of oxytetracycline is inactivated due to interacting factors and that it is unlikely this route of administered could facilitate absorption of sufficient concentrations of active chemical to treat a systemic infection (Teves-Brown, 2000). Lunestad & Goksøyr (1990) suggests that only about 5% of oxytetracycline present in sea water may exists in the free form, with the remainder bound in complex with magnesium and calcium. This would result in an estimated achieved oxytetracycline concentration of approximately 1 mg/L in treated tanks during the 2018 APF hatchery season. It is unclear if this treatment alone would be sufficient to eliminate or prevent infection of a potentially harmful bacteria (e.g. *Vibrio* spp.) given minimum inhibitory concentrations (MIC) for control is often reported to be much higher, at 13-14 mg/L (P, Rani J, & DR, 2018).

The exact mechanism behind the clinical benefit observed in the hatchery tanks during the APF 2018 season is not known. It is possible the administration of oxytetracycline via in-water immersion reduces the overall bacterial quantity in the system or assemblage of species present, this in turn may preventing overwhelming infections to a compromise host animal and/or facilitating recovery of the cultured prawn larvae.

Erythromycin is the other antibiotic that is used by some hatcheries in the Australian prawn industry with survival benefits in early larval rearing reported. Prophylactic and metaphylactic treatment in larval rearing using erythromycin in is not uncommon in prawn hatcheries, globally (Uddin & Kader, 2006). Increased erythromycin treatment (5 mg/L) was not found to be significantly associated with reduced risk of the case definition (0% survival) in the epidemiological analysis. The mechanism that erythromycin treatment has in a larval rearing system to reducing occurrence of larval Vibriosis is unclear, given the low dose used and the inherent resistance of gram-negative bacteria (e.g. *Vibrio* spp.) to erythromycin which has been reported in many such organisms to macrolide antibiotics (Akinbowale, Peng, & Barton, 2006). A significant proportion of *Vibrio* species (34%) isolated from in Australian aquaculture sources were found to be resistant to erythromycin (Akinbowale, Peng, & Barton, 2006). It has been suggested that the possible beneficial role of erythromycin in the larval rearing system relates to changes in the microbiome of within the prawn larvae or culture tank (Ian Anderson. Pers. Comms.).

The analysis performed in this project suggested the use of OTC (20mg/L) immersion treatment course (3 days) may increase the odds of survival through larval rearing. Efforts to understand and mitigate the cause of proliferation of potentially overwhelming *Vibrio* and other bacterial species

should continue, so that use of antibiotics can be minimised or eliminated altogether. Maintenance of high levels of sanitation and balanced feed provision in hatcheries remains critical to help prevent the creation of an environment which is prone to bacterial proliferation/overgrowth.

Additional identified potential risk factors

Potential risk factors spanning a whole range of hatchery variables were identified in the project (see Appendix 2), including: water quality factors, nutritional factors, additives (e.g. probiotics), chemicals/therapeutics, and husbandry practices (See 4.1.1. and Appendix 2). Although these risk factors may have contributed to the mortality events experienced by the APF hatchery, they were found to either, fall out of statistical significance throughout the epidemiological analysis process, or were not analysed due to gaps in available data. Reasons that variables fell out of statistical significance included: effects of collinearity, strong correlations, influence of the very strong run effect (temporal effect) linked to stocking sequence, and influence of random and mixed effects. The stage at which the above risk factors were identified to be no longer significant is reflected by the significance classification attributed to the potential risk factor. This allows for a hierarchical ranking of these apparent, but non-statistically significant variables as potential risk factors in these outbreaks. This in turn supports their consideration in future investigations, or, additional analysis. Some of these risk factors were assessed in relation to available literature to gauge whether they were considered relevant.

Sub-optimal water quality and nutrition were identified as potential issues during the initial field mortality investigation. Many potential risk factors within these two categories were addressed by hatchery staff during the season. This action did appear to result in improved growth, however, mortality events appeared to continue. Further focus on these identified potential risk factors may be warranted.

TAN, NH_3, pH, water temperature, and salinity within larval rearing tanks were found to be outside of recommended reference ranges for *P. monodon* (FAO Fisheries and Aquaculture Department, 2007; Appendix 2).

Sub-optimal feed type, abundance and frequency of application were identified in larval rearing tanks. Prawn larvae from multiple tanks were found to be in poor condition, low feed intake and reduced digestive activity (both before and after feeding). FAO Fisheries and Aquaculture Department, (2007) highlight the importance of feed presentation and feeding level in larval rearing, with timing and frequency of feeding highlighted. There are consequences from designing feeding practices around the convenience of the hatchery technician rather than to the behavioural feeding requirements of the species (FAO Fisheries and Aquaculture Department, 2007). Feeding larval prawns once or twice a day, during daylight hours, instead of distributing their feed at regular intervals during the night from dusk to dawn does not reflect the typical nocturnal feeding behaviour of the species (FAO Fisheries and Aquaculture Department, 2007). In addition, there has been found to be rapid loss of water-soluble nutrients (e.g. 20% loss of dry matter/ crude protein and up to 95% loss of water-soluble vitamins) through leaching from a conventional pelleted prawn ration after a one hour immersion period (FAO Fisheries and Aquaculture Department, 2007).

Detailed information regarding water quality and nutrition parameters was not available for each hatchery tank to allow them to be included in the epidemiological data analysis. Recommendation to reduce the potential risk associated with these factors is detailed in section 8.0.

Attention to these potential risk factors may offer benefits from a holistic approach to improving hatchery practices and produce a more robust animal, which may in turn improve tolerance and resilience to opportunistic and overwhelming infections, such as Vibriosis (See Section 8.1. for associated recommendations).

No Association Factors

Many potential hatchery risk factors assessed were found to be not significantly associated with the case definition (0% survival). Further detail regarding these factors can be found in Appendix 2 and Section 4.1.1.

There was no significant association between the presence of ciliates parasites and the case definition, which support the field assessment performed during the mortality events (See. 4.4; 5.4).

There was no significant association between the presence and relative abundance (CT value) of any of the three toxin genes (YaFO, RtX, and zon occludens) in routine pre-stocking hatchery PL samples and case definition (0% survival) or cumulative mortality. This relationship was assessed when adjusting the case definition to 10%, 20%, 30% and 40% with no change to the overall assessment outcome (data not shown). The relationship between toxin gene abundance (CT value) in routine pre-stocking hatchery PL samples and cumulative mortality in hatchery tanks was not different from zero. The presence or abundance of YaFO, RtX, or zon occludens toxin genes did not appear to have effected survival within the hatchery.

There was no significant relationship between the case definition (0% survival) and any potential primary pathogen detected in the hatchery analysis.

5.1.2. Grow-out Ponds

Three risk factors were found to be significantly associated with the case definition (0% survival) throughout all statistical analysis and comparisons performed on the grow-out data set. These included: Stocking grow-out ponds less than 11 days after completion of filling; maximum *Nitzschia* sp. score higher than 5 (0=absent; 5=low; 10=medium; 15=high) during the first 30 days post stocking; and grow-out ponds that had a minimum salinity of less than 36 ppt during the post stocking period (6 weeks), noting that where this occurred it appeared a consequence of significant rainfall events occurring after the time of filling and stocking the ponds (See appendix 1).

Time between initial pond filling and stocking larvae

The number of days between completion of initial pond filling and stocking PLs into the grow out ponds was reduced in response to hatchery mortalities. It was logistically challenging for the grow-out pond farm to prepare, fill and stabilise ponds in the face of unforeseen delays to availability of PLs for stocking. This practice did not prove to be effective with only 27% (4/15) of grow-out pond stocked with PLs, less than 11 days following initial pond filling, surviving to harvest. It is possible that this practice may have contributed to generating a less stable pond environment for culture, and less desirable feed resources for early prawn larvae. Ponds stocked with PL less than 11 days following initial pond filling, had apparent water quality differences during the post stocking period, relating to: pH (higher daily average, higher daily maximum, higher daily change), DO (higher daily average, lower daily minimum, higher daily change), Salinity (lower average, higher change), temperature (higher daily average, higher daily maximum), algal species (increased *Gymnodinium* sp. and *Nitzschia* sp, lower mixed dinoflagellates).

Early life stages of crustacea are particular susceptible to water parameter derangements and fluctuations (e.g. pH) (Boyd & Tucker, 2014). It is recommended that earthen aquaculture ponds should be prepared as early as possible, preferably several weeks before stocking to allow the initial flush of plant growth and water quality to stabilise before animals are introduced (Boyd & Tucker, 2014). The analysis performed in this project suggested that the time between pond filling and stocking should be greater than 11 days to increase the probability of grow-out survival.

Maximum Abundance of Nitzschia species

Routine algae monitoring is performed every 3-4 days at APF to assess relative abundance (0=absent; 5=low; 10=medium; 15=high) and species in each grow-out pond. Epidemiological analysis identified that both the maximum *Nitzschia* species score higher than 5, and an average *Nitzschia* species score of 2 or more, during the post stocking period (30 days), to be significantly associated with the case

definition (0% survival). These two variables were found to be highly correlated (Pearson's correlation >0.9), with the maximum *Nitzschia* spp. score of higher than 5 was found to have a stronger association (more highly significant) with the case definition. This risk factor was found in 96% of case ponds (26/27) but only 25% of non-case ponds (5/20).

Nitzschia spp. are a very common marine diatom with a large number of different species that are often difficult to identify. It is not typically considered a harmful algal species (Hallegraeff, 2015). It has been suggested that some *Nitzschia* spp. (*Nitzschia navisvaringica* and *N. bizertensis*) can produce the neurotoxin domoic acid (DA) (Bates, Hubbard, Lundholm, Montresor, & Leaw, 2018). This was discovered in prawn culture in Vietnam, although no prawns were reported to be toxic (Bates et al., 2018). Some *Nitzschia* ssp. have been known to reach great abundance in waters high in organic pollution (Spaulding & Edlund, 2008). The association between *Nitzschia* spp. and non-survival in the grow-out farm may not necessary relate to the algal species being directly harmful, but could reflect that this species is commonly found in an less stable or sub-optimal pond environment The analysis performed in this project suggested that when elevated levels of *Nitzschia* ssp. were detected in the post stocking period (30 days) there was a decreased probability of survival through grow-out. Extended periods of elevated *Nitzschia* ssp. should therefore be avoided to increase the probability of survival through grow-out. Increasing water exchange, addition of fertilisers or approved chemical products (e.g. algicidal agents) to shift the pond water quality or algal species assemblage should be considered.

Pond salinity in the post stocking period

Water salinity was not frequently monitored in grow-out ponds during the 2018/19 production. Analysis of the data that was available from each grow-out pond during the post stocking period (6 weeks), when three or more measurements were performed, revealed a significant relationship to non-survival (elevated mortality). Epidemiological analysis identified that both the minimum salinity of less than 36 ppt, and maximum salinity change of 6 ppt or higher, during the post stocking period (6 weeks), to be significantly associated with the case definition (0% survival). These two variables were found to be closely related to each other. Comparative mixed variable analysis determined that the risk factor of minimum salinity of less than 36 ppt to have a more strongly significant association with the case definition (0% survival). This risk factor was found in 93% of case ponds (25/27) but only 15% of non-case ponds (3/20).

Salinity (alongside temperature and pH) have been shown to significantly influence the moulting and growth of *Penaeus indicus* (Vijayan & Diwan, 1995). At salinities of 15 and 25 ppt prawns moulted normally, whereas at 35 and 45 ppt salinities prawns showed an extended moult period and decreased feed intake (Vijayan & Diwan, 1995). Outside optimal salinity, pH and temperature, feed intake, normal moulting, and resulting growth was impacted (Vijayan & Diwan, 1995).In extreme salinities prawns must expend considerable energy to maintain osmoregulation (Vijayan & Diwan, 1995). Dramatic salinity changes are considered to add a large physiological stress on the animal. Salinity is considered the most important abiotic factor affecting the growth and survival of penaeid prawns except for temperature (Su, Feng, & Ma, 2010). Some prawn health benefits have been reported to mild salinity fluctuations and consequences for larger salinity fluctuations (Su et al., 2010). At 30℃ *L vannamei* exposed to a salinity fluctuations over a 15 day cycle period of ±10 or ±15 ppt had a significantly higher mortality than *L vannamei* exposed to a salinity fluctuations of ±0 or ±5 ppt (Su et al., 2010). Nutrition impact the ability of prawns to withstand salinity changes. Jory, (2018) suggested that larval prawns fed a diet with high HUFAs (highly unsaturated fatty acid) to have improved ability to withstand salinity changes during post stocking acclimatisation and transition to grow-out systems after stocking. It is possible low or large changes in salinity may have a direct, or indirect, or a combination of effects in post stocking prawns. Some fluctuation is likely unavoidable, however low salinities and larger fluctuations should be avoided to increase the probability of survival through grow-out. Creation of a hyper salinity water storage may offer one option to help moderate salinity drops. Routine salinity monitoring, increasing or decreasing water exchange, and investment in expansion of post-pumping water storage or farm water recirculation capacity should be considered.

Additional identified potential risk factors

Potential risk factors spanning a whole range of grow-out variables were identified in the project (see Appendix 3), including: algal species and abundance, water quality factors, chemicals/therapeutics, husbandry and infrastructure factors, and pond preparation and management practices were identified in the project (see 4.1.2. and Appendix 3).

Stocking density appeared to have an inverse relationship on grow-out survival (e.g. pond stocked at a higher density were more likely to survive and had a higher average survival at harvest). However, stocking density of less than 70 PL per sqm was not found to significantly associate the case definition (0% survival) due to the very strong temporal influence on mortality. Earlier stocked grow-out ponds were more likely to have prawns survive to harvest (e.g. not meet the case definition) (see appendix 1).

Elevated pH (average and daily pH fluctuation) and total ammonia nitrogen (TAN) were highlighted as potential risk factor from early field observations. These water quality factors did not remain significantly associated with the case definition through the entire epidemiology analysis, due to correlations with more strongly associated risk factors (e.g. time between pond filling and stocking). However, these factors are still considered important when seeking to optimise environmental conditions for cultured animals. Cui et al., (2017) suggested that prawns exposed to elevated ammonia nitrogen might demonstrate weakened metabolic and immunological responses. While acute (72 hour) high (pH 9.7) or low (pH 6.9) pH stress has been shown impair the intestine barrier function of *L. vannamei*, likely via destroying mucosa structure, disturbing digestion and metabolism, inducing oxidative stress, disordering immunity, and disruption of the microbial composition (Duan, Wang, Liu, Zhang, & Xiong, 2019). High pH and high unionized ammonia level of culture water were identified as stress factors that lead to disrupt the immune ability and metabolic performances of prawns increasing the susceptibility to microbial infections (Joseph & Philip, 2007).

Interestingly, stocking PLs of a younger age at stocking did not appear to remain significantly associated with the case definition (0% survival). There was an apparent increase in mortality (non-survival) in ponds stocked with young PLs, however, this was not found to remain significant, due to the strong temporal influence on mortality. It is possible that this finding is a result of limited data of older PL being stocked latter in the season, or PL age did not appear to be a major variable contributing to risk of mortality in this scenario. It is still recommended to stock larger size and aged PLs, as per guidelines (FAO Fisheries and Aquaculture Department, 2007). There are many factors that could contribute to reduced survival when stocking younger PLs such as temperature, salinity, pH and ammonia (Magallón Barajas, Servín Villegas, Portillo Clark, & López Moreno, 2006; (Tsuzum, Cavalli, & Bianchini, 2007). Stocking PLs between PL15 and PL20 has been found to have improved survival rates (Tsuzum et al., 2007). This could be achieved using a use of a nursery system to rear larger and older PLs for stocking. The stocking of early stage PL's places greater reliance on the pond's natural food production to support early nutrition. Hence it has been reported that reliability of stocking improves with the stocking of older, larger larvae.

See section 5.1.1 for more information of assessment of risk factors and attributing significance. Considering these potential risk factors may still offers benefits from a holistic approach to improving grow-out practices and produce a more robust animal, which may in turn improve tolerance and resilience to opportunistic infections, such as vibriosis. See Section 8.2 for associated recommendations.

No Association Factors

Many potential grow-out risk factors assessed were found to not significantly associate with the case definition (0% survival). Further detail regarding these factors can be found in Appendix 3 and Section 4.1.2.

There was no apparent link between hatchery performance (average, maximum or minimum survival) and survival in grow-out ponds, with no significant association to the case definition (0% survival) found. It should be noted that only hatchery (larval rearing) tanks that survived could be analysed,

therefore a survival bias is likely influencing the available data and therefore interpretation. In any-case, no link between mortality in the hatchery and grow-out production could be found. It is considered that these mortality events may reflect independent and mutually exclusive disease events that could be linked by factors within individual animal unit (e.g. host factors), or similar environmental factors (e.g. sub-optimal water quality), but not common infectious pathogen factors (e.g. virus, primary bacterial pathogen). It did not appear that an infectious pathogen was transferred from the hatchery to grow-out ponds in the assessment of the current data set. The capture of additional health assessment information around stocking of PLs into grow out ponds would allow further understanding of the relationship between apparent PL health and subsequent grow-out performance.

Health score or stress test score did not appear to significantly associate with the case definition (0% survival). This suggests that mortality may have not occurred in the post stocking acclimatisation or first few days, provided the APF health score or stress test have a sufficient sensitivity to detect a health issue prior its occurrence at this stage of production. The use of a stress test involving exposing a sub-sample of PLs to reduced salinity (e.g. 50% lower salinity) for 2 hours provides a simple and rapid test for assaying the physiological condition of PLs (FAO Fisheries and Aquaculture Department, 2007; Rees et al., 1994). Further recommendations are provided (See Section 8.2) to further improve these techniques to increase the value or usefulness of the information outputs.

While there was an apparent increase in mortality (non-survival) in ponds stocked with young PLs, this was not found to be significant, due to the strong temporal influence on mortality.

The use of oxytetracycline (20mg/L immersion treatment) or erythromycin (5mg/L immersion treatment) treatments in the hatchery did not significantly associated with the case definition (0% survival). This suggests that the use of these therapeutics did not appear to have a positive or negative legacy effect on survival of treated animals beyond exposure in the hatchery and outcomes within the larval tank.

There was no significant association between the presence and relative abundance (CT value) of any of the three toxin genes (YaFO or RtX) in routine pre-stocking hatchery PL samples and case definition (0% survival). The presence or abundance of YaFO or RtX toxin genes did not appear to have effect survival in grow-out ponds. This result differed for zon occludens toxin gene abundance (CT value $\geq$34) in routine pre-stocking hatchery PL samples which was found to have a marginally significant ($p<0.01$) association to the case definition (0% survival). It is perhaps counterintuitive that increased CT value (lower toxin gene load) had an apparent relationship with decreased survival. This association was no longer found to be significant when comparing confounding variable interactions which identified collinearity effects. Therefore, complex analysis demonstrated the relationship was not significant.

There was no significant relationship between the case definition (0% survival) and any potential pathogen detected in the hatchery analysis.

5.1.3. Prospective Trial Ponds

Comparison to commercial grow-out ponds

All three of the prospective trial ponds did not meet the case definition (0% survival). However, majority of all three trial ponds appeared to align with the three risk factors that were found to be significantly associated with case definition in the commercial data retrospective epidemiology analysis. These included: stocking grow-out ponds less than 11 days after completion of filling and maximum Nitzschia sp. score higher than 5 during the first 30 days post stocking (except for pond 45). There may be several explanations for this unexpected outcome, including influence of one of the highlighted limitations of the epidemiological analysis (See 5.1.4), or a limitation in the prospective pond trial design (See below and in section 5.5.6). These findings provide further support to the potential relevance of the identified salinity risk factor (See 4.1.2). Given the trial ponds did not meet the case definition further detailed epidemiological data analysis was not performed.

Comparison to within trial grow-out ponds

Although the three trial ponds did not meet the case definition (0% survival), and apparent survival difference was observed between the ponds, with pond 44 having a survival greater (approximately three times greater) than pond 43 or pond 44. Differences between these two groups are highlighted in 4.1.3. Only mild-moderate differences were apparent. No obvious differences were determined that could explain the survival difference. See sections 4.3 and 5.3 for further information regarding differences between ponds. Given the low sample number in each group (n=1, and n=2) if is not possible to determine the statistical significance of any observed or suspect risk factor between these two groups.

5.1.4. Epidemiology Analysis Limitations

The above discussed risk factors, and the extended list of potential risk factors (See appendix 2 and 3), are not considered to be likely stand-alone drivers of the case definition (0% survival) but reflect a range of potential co-contributing variable that strongly-weakly associate with the occurrence of the case definition (0% survival). This analysis merely assesses the distribution or spread of the data, assesses patterns that may exist, and seeks to determine significance (or lack of) to the relationship between a potential variable and the case outcome. This can then allow for prioritisation and targeting of management measures to increase the probability of the highest level of risk reduction. The use of this framework can be continually reviewed to strengthen the understanding between a range of risk factors (explore) and the case outcome (0% survival), using the best available evidence. No single identified risk factors, alone, is considered likely to cause (or prevent) mortality, completely. There are likely many other potential risk factors that were not included in the statistical analysis may, at least in-part, contribute to the observed effects.

Equally there are likely many confounding factors that were able to be not assessed which could have contributed to the survival outcome to a lesser/ equal/ greater extent as assessed risk factors. This is in-part due to the necessary management responses during a disease event in order to prevent further stock loss, and in-part due to gaps in available data which shaped the available analyses. Some of these changes that occurred over time included changes to: tank disinfection protocol, live feed (e.g. reduced algae culture water addition to larval tanks, washing and concentrating of live algae, use of blanched and live artemia, alterations to feed frequency), husbandry (e.g. changes to tank stocking sequence, introduction of health assessments and increased health surveillance), monitoring and management of water quality (e.g. timing of water exchange, reducing periods of sub-optimal pH, salinity, and ammonia), use of probiotics, and facility cleaning and disinfection practices.

Additionally, there was a limited number of ponds available to make up the data set in the grow-out data set (47 ponds total), this may add increased error in the analysis outputs and restrict ability to determine significance of individual or multiple variables.

It is anticipated that the potential risk factors associated the case definition will change as additional information becomes available, farm practices change, disease profile/expression changes, the case definition is further refined and/or overall animal health and robustness is improved.

The use of a case definition based on mortality, alone, has its limitations. Given the multitude of clinical syndromes that are likely to exist either within the hatchery and grow-out stages of a prawn farm, which may cause mortality, there is an inherent level of error expected associated with this approach to assessment of the data. Refinements in the case definition were unable to be made as no consistent pathology or clinical signs were apparent in affected prawns.

The selection of 0% survival as the definition of a case was made to try and identify with the greatest clarity, the largest contributors to the observed problems. The presumption was that, should there be the involvement of a pathogen, or other risk factor, causing the mortality, then it is most likely to be present in the most severely impacted ponds, and identified by the statistical analysis using this approach. In addition, the sample size of the non-case populations in the available grow-out data set

was small (n=20), with a case definition of less than 30% survival, the sample size is further reduced (n=6), which would further weaken the statistical analysis.

5.2. Development of a qPCR for bacterial toxin genes identified in Next Generation Sequencing (NGS) of bacterial cultures collected from APF water during the 2018 hatchery season

5.2.1 Next Generation Sequencing (NGS)

The use of NGS successfully identified three toxin gene targets from hatchery tank samples experiencing elevated mortality: zon occludens, RTX, and YaFO. The presence of these genes in prawn culture environments was perhaps not unsurprising as they have been demonstrated to be associated with *Vibrio* spp. This facilitated further investigation into diagnostic tests to detect the presence and relative abundance of these toxin genes within prawn tissue samples (see 5.3.).

The results highlighted limitations in the sample type (ethanol fixed PL's) that was used for NGS. It was suggested by JCU AquaPath lab that high levels of host DNA present in the sample, may have limited the ability for NGS to detect RNA pathogens, such as GAV which were considered likely to be present in the samples given the previous PCR testing results. It was also suggested that in future freezing of samples for NGS, rather than fixed in ethanol, would assist in trialling different methods of processing.

The methodology used for NGS would be improved if a method of external decontamination could be developed. This would potentially allow differentiation of internal genetic material and external/surface genetic material. Conrads & Abdelbary (2019) suggested that for unbiased NGS-results a contamination-free collection, homogenization, storage and efficient DNA extraction is essential.

The use of mixed bacterial cultures from a TCBS agar plate infused with erythromycin, as the NGS test sample material, may cause a selection pressure on the isolated bacteria that could potentially generate results that are misleading and not truly reflective of disease pathophysiology impacting the host animal. An alternative approach to consider might be using heavy growth single colony isolates collected using an aseptic approach to sterile saline and ethanol rinsed homogenised PLs and cultured on a non-selective agar media. This may eliminate some of the factors mentioned above that could potentially impact the NGS outputs.

NGS has been touted as a revolutionary new tool to assist investigating unknown infectious diseases (Chai et al., 2018; Gwinn, MacCannell, & Armstrong, 2019). This of course has limitations when investigating non-infectious unknown diseases. NGS can increase speed, accuracy, and detail but also increases cost of infectious disease investigations (Gwinn et al., 2019). A cost-benefit review of the use of NGS in unknown disease investigations suggested current conditions do not warrant a widespread rush to deploy NGS to resolve any and all uncertainty, but rather as a front-line technology that should be used in specific contexts, as a supplement to rather than a replacement for careful clinical judgement (Chai et al., 2018).

5.2.2. Development of toxin gene qPCR

JCU Aquapath lab successfully developed a qPCR test for all three toxin gene targets (zon occludens, RTX, and YaFO) generated from the NGS outputs. This facilitated further investigation into the relevance and association of these toxin genes to hatchery larval rearing tanks and grow-out ponds that experienced elevated mortality during the 2018/19 production season.

Methodology standardisation

The methodology for sampling was not standardised between 'routine' hatchery PL samples collected pre-stocking into grow-out ponds, and 'opportunistic' hatchery PL samples collected during a mortality event detected in the hatchery, and during health assessments of the prospective trial ponds.

Therefore, quantification of these unstandardized samples is not relevant. It is not possible to determine whether the quantity variation in the test output (e.g. CT value) is due to in test methodology differences or true population differences.

5.2.3. Repeatability of the toxin gene qPCR

The accuracy of a test procedure can be measured using either assessing validity or precision (Sergeant & Perkins, 2015). The importance of understanding test validity is paramount. Many factors may contribute to the variability of a test procedure, including; uniformity of test material, transport and storage of test material, reagents, equipment and its calibration, operator, and the environmental conditions (e.g. temperature, humidity, light) (Sergeant & Perkins, 2015). Precision is a general term used to describe variability between repeated tests on apparently identical material. A test with a high-level precision has a low variability and vice versa (Sergeant & Perkins, 2015). Precision can be accessed via the repeatability (consistent results on repeated replicates of the same sample material – minimum variability) and reproducibility (consistent results on repeated tests of the same sample material – maximum variability) (Sergeant & Perkins, 2015). An assessment of the repeatability of the developed qPCR toxin gene tests was performed to assist understanding how best to extrapolate of the data outputs from these diagnostic tests within this project.

The consistency of detection

The consistency of detection was found to be very high for the three-toxin gene qPCR tests. It was clear that for this type of sample (where the test target is expected to be in large quantity), the detection results were very consistent, with greater than 95% of PL batches tested found to have perfect agreement with all 5 test replicates, and only rare instances (<5%) where discrepant detection occurred between the five sample test replicates. This suggests that in this case, very little information may be lost when the number of test replicates are reduced. Therefore, for the purpose of toxin gene detection in a PL population samples, testing fewer replicates could improve economic use of resources. Further analysis would be required before determining if this outcome could be replicate in other testing methodologies.

The consistency of quantification

The consistency of quantification was found to be very high for the three-toxin gene qPCR tests. CT values were found to be very close between test replicates of the same PL batch despite the whether the analysis included all five replicates, or only four, or only three, or only two replicates, in all possible combinations. With the full dataset (all 5 replicates), the largest repeatability coefficient was observed with YaFO gene replicates with a value of 3.06. That is to say that 95% of replicated CT values within a sample for YaFO quantification are expected to be within 3 cycles from each other. This is considered within the expected variability of real-time PCR quantification (Pers. Comms. Charles Caraguel). If the number of replicates was reduced, expectedly the repeatability reduces in extreme cases (minima) which suggests a loss of data variability. However, this is an exception and not the rule (maxima captured a lot variability) as most combinations of reduced number of replicates remained approximately close to the estimate with 5 replicates. This suggests that in this case, a reduction of replicates is unlikely to result in a major loss of information for quantification. Reducing replicates to three (triplicates), at most, would provide equivalent test output information. If resources are limited, duplicates would reflect fairly-well the variability too, for this type of sample (where the test target is expected to be in large quantity). The change can result in a cost saving of approximately 60% (current project testing). Sample replication may be more justified when target concentration is scare (e.g. screening apparently healthy animals for certification purpose).

5.3. The presence of putative bacterial toxins in PL samples from APF larvae tanks experiencing elevated mortality during the 2018 hatchery season

5.3.1. Toxin genes (YaFO, RtX, and zon occludens)

There was an apparent increase in the abundance all three toxin genes (YaFO, RtX, or zon occludens) in hatchery PL populations experiencing elevated mortality at the time of sampling, compared to routine samples collected from PL population that did not have elevated mortality detected. However, this comparison is confounded due to the methodology for sampling not being standardised between the routine and opportunistic hatchery PL populations. Quantification of these unstandardized samples is not able to be assessed, as it is not possible to determine whether the quantity variation in the test output is due to in test methodology differences or true population differences. In addition, the mortality PL population sample would be expected to contain numerous dead larvae and thus a greater abundance of bacteria due to post-mortem colonisation. The presence these bacteria (and toxin genes they may carry) does not allow inference of causation.

The epidemiological data analysis did not find a significant association between the presence or abundance of YaFO, RtX, or zon occludens toxin genes from routine hatchery PL batch samples and the case definition (0% survival) or cumulative mortality in either the hatchery or grow-out APF production (See Section 5.1.1). These three toxin genes therefore did not appear to effect survival in the hatchery or grow-out prawn populations.

In the current available literature YaFO, RtX and zon occludens toxins do not appear to have been found linked to any previous disease in penaeid species. RtX (repeats-in-toxin) toxins were described in 1985, and has since been shown to have nodulation related, protease, lipase, heme-binding or bacteriocin activities against many different cell types from a wide variety of species (Welch, 2000). In *Vibrio cholerae* the MARTXVc RtX toxin gene is found in practically all strains (including environmental and clinical isolates and pandemic strains) (Pérez-Reytor, Jaña, Pavez, Navarrete, & García, 2018). YaFO toxins were described in 2009, and since been shown to act by inhibiting protein synthesis in *E.coli* (Y. Zhang, Yamaguchi, & Inouye, 2009). No literature on YaFO toxins in *Vibrio* species were found. Zon occluden toxins were described in 1991, and has since been shown to act by attacking cell junction integrity to increase mucosal cell permeability (Di Pierro et al., 2001). Zon occludens toxin gene has recently been isolated from virulent AHPND causing *Vibrio parahaemolyticus* strains (Lee et al., 2015; Prachumwat et al., 2019). However, it was not yet determined whether zon occludens was toxic, or enhanced virulence of AHPND toxins, or had influenced disease expression caused by these bacterial strains (Prachumwat et al., 2019). Amongst well described prawn toxins from *Vibrio* species, which are known to cause high mortality in shrimp there is some ambiguity in the underlying triggers for expression and function of these toxins. Recent literature has shown that *Vibrio parahaemolyticus* strains containing PirvpA and PirvpB toxin genes and producing PirA and PirB toxin do no always cause high mortality or pathognomonic AHPND lesions (Lai et al., 2015).

RtX and zon occludens were first detected in routine PL population samples from mid-way through run 1, before all three toxin genes (YaFO, RtX and zon occludens) appeared widespread from run 3 onwards. This trend in toxin gene presence may reflect bacteria presence and abundance overtime in the hatchery. Bacterial assemblages in a commercial shrimp hatchery are typically lowest at the start of the production season and highest at the end of the production season. Kumara & Hettiarachchi (2016) found that mean total *Vibrio* sp. counts increased throughout the prawn larvae rearing production cycle lowest to highest in: disinfected water < algae tanks < incoming water < nauplii < Mysis < PL15. Broodstock maturation tanks, spawning tanks, artemia tanks and PL15 tanks had the highest mean total *Vibrio* sp. counts (Kumara & Hettiarachchi, 2016). Therefore, it may not be surprising that bacteria detection becomes more widespread towards the end of hatchery season, particularly where there has not been an interval for complete dry-out of the system. There was no length of time where the entire APF hatchery was empty, cleaned and disinfected during the season

before the next larvae run, there was only between 0-4 days length of time where an entire rearing room of the APF hatchery was empty, cleaned and disinfected during the season before the next larvae run, so persistence of bacteria within the facility appeared plausible.

This project does not appear to have changed the status of these toxins, from general bacterial products which bacteria use to assist general function, to factors that significantly associate with causing or contributing to disease in penaeid species. The three toxin genes explored in this project (YAFO, RtX, zon occludens) did not appear to contribute to the mortality events encountered in the hatchery and grow-out production. This is a useful outcome for assisting future disease investigations that involve *Vibrio* species.

Comparison between hatcheries

The presence of all three toxin genes (YaFO, RtX, and zon occludens) in other Australian prawn industry hatcheries that did not experience equivalent mortality events in the hatchery and grow-out production, as the 2018/19 APF season, suggested these toxin genes do not appear to be unique to the APF hatchery and have not yet been proven to be causative in driving mortality in hatchery and grow-out ponds. This aligns well with the project findings.

Alternative bacterial products

It is possible that an alternative toxin, which was not identified in the current project, contributed to the mortalities in the hatchery and grow-out ponds. However, in absence of further information this was not able to be confirmed. It is considered that pathogenic *Vibrio* species produce various virulence factors including enterotoxin, haemolysin, cytotoxin, protease, lipase, phospholipase, siderophore, adhesive factor and/or haemagglutinins (X. H. Zhang and Austin, 2005). These are bacterial products which typically assist invasion, immune evasion, colonisation and proliferation. Haemolysin, which is an exotoxin that lyses erythrocyte membranes with the liberation of haemoglobin (X. H. Zhang and Austin, 2005). Haemolysins are considered one of the most widely distributed toxins among pathogenic *Vibrio* species (X. H. Zhang & Austin, 2005). So much so that they have been found to be useful identify *Vibrio* species. A study by Torky & Ghanem (2017) found that testing for the a *Vibrio harveyi* haemolysin gene (hly) was an accurate and fast method to detect *Vibrio harveyi* (highly pathogenic, moderately pathogenic and non-pathogenic strains) due to the presence of a single copy of haemolysin gene encoded in all isolates (Torky & Ghanem, 2017).

These findings suggest that, within a genetic test framework, it may not always be possible to differentiate a test output as reflecting presence of abundance of a toxin or presence of abundance of bacteria.

Diagnostic Considerations

There are many potential non-valid assumptions that can arise when using a genetic test (e.g. PCR) to detect (presence/absence) or quantify (CT value) a genetic sequence target (e.g. toxin gene) and attribute a causal association with an outcome (e.g. mortality) or pathological condition observed in the animal test subject.

Firstly, presence or abundance of a bacterial toxin gene (or genetic material) does not necessarily relate to presence or abundance of an expressed toxin protein. Secondly, presence or abundance of a bacterial toxin gene (or genetic material) does not necessarily relate to disease incidence or severity. The above two concepts were demonstrated by Rattanama et al., (2009), when investigating the potential pathogenicity of a hemolysin toxin genes in *Vibrio harveyi*, isolated from shrimp that died from vibriosis in Thailand. They found that although the hemolysin toxin gene was detected in all samples not all the isolates caused haemolysis (Rattanama et al., 2009). Bacterial isolates that were positive for the toxin gene did not reliably cause haemolysis or mortality (Rattanama et al., 2009).

Thirdly, presence or absence of a bacterial toxin gene (or genetic material) may relate merely to presence or abundance associated bacteria that commonly carry that genetic material. The above concept was highlighted by the Torky & Ghanem (2017) study discussed above.

Fourthly, presence or abundance of a bacterial toxin gene (or genetic material) does not necessarily relate to presence or abundance of this material within a living organism (e.g. prawn larvae). In absence of appropriate surface disinfection, the presence or abundance of a bacterial toxin gene (or genetic material) may relate to toxin genes (or genetic material) external to the target animal (e.g. in the water that a PL may be held in).

Lastly, presence or abundance of a bacterial toxin gene (or genetic material) does not necessarily relate to presence or abundance of this material within living bacteria. PCR does not have the ability to differentiate bacterial toxin gene (or genetic material) from living and dead bacteria.

The above factors need to be considered when interpreting a toxin gene molecular test. Inference of disease to a population level should not be made using molecular data alone. It is critical to note that molecular typing and genomic data, however advanced and detailed they may be, cannot compensate for sound epidemiological design of surveillance (Stärk, Agnieszka, & Muellner, 2019). Samples need to be representative of the population about which conclusions will be drawn and of a sufficient sample size (Stärk et al., 2019). When samples are collected, additional epidemiological information needs to be captured (so-called meta-data) to allow for an appropriate interpretation at population level (Stärk et al., 2019). Single isolates – even if analysed at nucleotide level – provide very limited epidemiological value in the absence of information on the target population from which they were drawn (Stärk et al., 2019).

This outcome contrasts with diagnostic results for other bacterial toxins such as the PirAB toxin. This toxin is considered to be involved in clinical causation of AHPND in SE Asia and is responsible for some of the observed Early Mortality Syndrome (EMS) reported in those cases (Sanguanrut et al., 2018). A recent study showed that only just over half of all EMS pond cases detected were due to AHPND, with almost one quarter of these cases positive for AHPND causing toxin genes (Sanguanrut et al., 2018). In addition, many of the pond cases displayed significant histopathological findings but did not test positive to the AHPND PCR or display pathology consistent with the AHPND cases (Sanguanrut et al., 2018). These findings support the need for full detailed disease investigations using multiple diagnostic modalities.

5.4. Potential pathogens involved in elevated mortality experienced in the hatchery and grow-out production stages at during the APF 2018/19 season

There were no single new, existing or emerging infectious organism identified in the investigations to be responsible for the low survival in APF tanks or grow-out ponds. This aligns with the field assessment throughout the mortality event investigation.

5.4.1. Hatchery

There was a gap in sample collection over run 2 where all hatchery tanks experienced elevated survival.

Parasitology

Epidemiological data analysis and field findings (including histopathology) suggested that the presence or relative abundance of ciliated parasites in larval rearing tanks did not appear to be driving the elevated mortalities experienced in the APF hatchery. These ciliate parasites were not detected in any of the affected were suggested to be hypotrich ciliates (Such as *Oxytricha* sp. or *Euplotes* sp.), which are common in the aquatic environments, associated with high organic loading conditions, and are rarely considered pathogenic (Peter O'Donoghue Pers. Comms.). Typical management strategies for these parasites include reducing organic content, avoiding overfeeding, improving water flow, improving water quality, and/or chemical control (e.g. formalin) (Peter O'Donoghue Pers. Comms.).

Virology

No potential viral pathogens, which could explain the elevated mortalities experienced in the APF hatchery, were consistently detected in broodstock surveillance samples, routine PL samples or opportunistic PL samples collected prior to and during the hatchery mortality events.

IHHNV and GAV were detected within some of the hatchery PL populations, however, were not consistently found in affected populations (50% or less). In addition, histopathological changes associated with disease attributed to these two endemic viruses were not apparently in submitted samples (For GAV pathology see Callinan et al. (2003); for IHHNV pathology see: Hsieh et al., (2006). The absences of CMNV, IMV, LsV, MoV, SHIV, TSV, WSSV, YHV-1 and YHV-7 detection in all assessed hatchery samples suggests it is very unlikely these potential viral pathogens were involved in causing the observed hatchery mortalities. No pathology was detected that would suggest the potential involvement of a novel virus.

Bacteriology and Mycology

Pathology from hatchery populations experiencing elevated mortalities during larval rearing suggested a potential role for likely mixed *Vibrio* spp. infections. Vibriosis is a common disease encountered in *Penaeus monodon* larval rearing with bacterial counts commonly found to increase over time in the larval population and sanitation and hygiene procedures critical for disease prevention and control (Torres, 2008; Aguirre-Guzmán et al., 2001). It was hypothesized that apparent increased incidence and severity of these infections were potentiated by a range of unidentified environmental stressors on the cultured larvae. Vibriosis is typically more common is early hatchery life stages (Sathish Kumar et al., 2017). However, in this instance prawns were affected at later larval stages of development throughout post larval stages (See Appendix 1). This was considered unusual in its presentation.

The histological finding of proliferation of bacteria in the organs and tissues of dead larvae was considered a post-mortem artefact. While proliferation of bacteria in the mid-gut and hepatopancreas with necrosis of mucosa with rounding and sloughing of cells, which was present in all submitted samples was considered significant and possibly due to initial bacterial infection progressing to widespread and lethal necrosis of MG and HP. These pathology findings are consistent with previous descriptions of prawns suffering vibriosis (Lightner 1993 In: Khimmakthong & Sukkarun, 2017). It was suspected that initial bacterial infection progressed to widespread and lethal necrosis of mg and hp. The drivers for this bacteria proliferation, severe bacteria enteritis and hepatopancreatitis were not clear, however a role of sub-optimal nutrition, sub-optimal larval rearing environmental conditions and husbandry, and potentially pathogenic bacterial species were suspected. Melanisation of external cuticle was noticed in all tank samples submitted and likely reflect an immune response of through activation of the prophenoloxidase activating system (proPO system) (Sritunyalucksana & Soderhall, 2000).

The lack of AHPND VpPirA, EHP, NHP or fungi detection in any hatchery tanks samples suggests it is very unlikely these bacteria or related organisms were involved in causing the observed mortalities.

Aseptically collected bacteriology samples from homogenised affected PL populations experiencing elevated mortality were absent from the available data, which limits the ability to assess whether a bacterial species was more consistently found in association with poor outcomes. A mix of *Vibrio* spp. and other marine bacteria that are considered typical environmental origin for prawn hatchery, were isolated. This included *Vibrio alginolyticus*. Kumar (2017) also found mixed *Vibrio sp.* when investigating elevated mortality in zoea stage *L. vannamei.* While the researchers found a higher association with *Vibrio alginolyticus* in the affected larvae, its role as pathogen could not be resolved since *V. alginolyticus* is also associated as natural flora of healthy larvae and considered an opportunistic pathogen in penaeid shrimp (Kumar, 2017). The opportunistic nature of these bacteria has also been suggested with some AHPND causing *Vibrio parahaemolyticus* strains. A study by Khimmakthong & Sukkarun (2017) exposed apparently healthy *L. vannamei* to an immersion of AHPND causing *Vibrio parahaemolyticus* for 1 hour. They immediately post exposure found inflammatory infiltration on histopathology, followed by detection of PirABvp toxin and apparent

widespread organ distribution of *Vibrio parahaemolyticus* until 6 hours post exposure (Khimmakthong & Sukkarun, 2017). However, following 6 hours post exposure, *V parahaemolyticus* was no longer detected via PCR in tissues, suggesting the potential clearance and/or destruction of these bacteria by the prawn immune system, while toxins appeared to continue to cause damage to the hepatopancreases until 72 hours post exposure or until death (Khimmakthong & Sukkarun, 2017). The ability for *L. vannamei* prawn larvae to survive an immersion challenge to differing doses of *Vibrio harveyi, V. parahaemolyticus, V. alginolyticus* and *V. penaeicida* has been demonstrated by Aguirre-Guzmán et al. (2001). Differences in the response of various prawn populations to factors triggering vibriosis has been suggested to be due to their individual capacity to resist stress influenced by their nutritional and physiological make-up (Lavilla-Pitogo, Leaño, & Paner, 1998).

The above findings suggest that is it likely the bacterial component of the hatchery mortalities was a related to overwhelming opportunistic bacterial (e.g. *Vibrio* sp.) infection of prawn larvae of a sub-optimal health status.

5.4.2. Grow-out

There were challenges obtaining samples partly due to the low survival and partly due to difficulties sampling prawn in the weeks following stocking into the grow-out ponds. As a result, there was a limited sample number able to be collected and assessed within each pond population and not all affected ponds were able to have testing performed.

Parasitology

Evidence parasites were only detected in one pond (pond 27) that was suffering elevated mortalities. However, this detection was in a near harvest pond from a population that were classified as a non-case, as the population survived to harvest and thus survival was greater than 0%. It is considered these sessile ciliate parasites are not related to the grow-out mortality events that occurred prior. Given no parasites were detected in any field microscopy or histopathology in affected pond populations during the initial investigations, which more closely reflect the disease events that occurred, it is unlikely parasites were a driving the elevated mortalities experienced in the APF grow-out.

Virology

No potential viral pathogens, which could explain the elevated mortalities experienced in the APF grow-out ponds, were consistently detected in diagnostic samples from pond population that experienced elevated mortalities.

IHHNV and GAV were detected within some of the prawns from grow-out PL populations, however, were not consistently found at high levels in affected populations (62% or less). In addition, histopathological changes associated with disease attributed to these two endemic viruses were not apparent in submitted samples (For GAV pathology see Callinan et al., (2003); for IHHNV pathology see: Hsieh et al., (2006)). Prevalence of IHHNV and GAV appeared to be high within one pond (27) that was experiencing mortality near harvest, however, again in these samples histopathological changes associated with disease attributed to these two endemic viruses were not apparent, suggesting they may not have been causing tissue damage. In addition, this pond did not meet appear to experience elevated mortalities (non-case pond) in the post-stocking period suggesting these results may reflect typical background viral levels. Further research using multiple modalities (e.g. gross pathology, histopathology, immunohistochemistry, molecular biology) with multiple replicates is needed to definitively determine the health impacts and further initial work on IHHNV (Sellars et al., 2019) and GAV (Munro, Callinan, & Owens, 2011; Callinan et al., 2003) in farmed *Peneus monodon* in Australian.

The histology findings of increased spheroids in early sample submissions could reflect increased viral, bacterial foreign material and/or cell apoptosis (Pongsomboon, Wongpanya, Tang, & Chalorsrikul, 2008; Van de Braak et al., 2002; Rusaini & Owens, 2010). It has been suggested that spheroid cells constitute a major site of viral degradation as parts of the non-specific prawn defence

system (Rusaini & Owens, 2010), and that spheroids are produced from spent granular haemocytes and eliminated to the environment rather than in situ destruction via necrosis or apoptosis (Rusaini & Owens, 2010). Presences of these structures has been associated with viral infections, such as GAV (Cowley, Hall, Cadogan, Spann, & Walker, 2002). In this investigation the detection of elevated spheroids did not appear to consistently align with elevated prevalence of IHHNV or GAV, in the small number of samples assessed. Therefore, the driver of the spheroids is not known.

The lack of TSV, WSSV, YHV-1 and YHV-7 detection in any grow-out pond samples suggests it is very unlikely these viruses were involved in causing the observed mortalities. No pathology was detected that would suggest the potential involvement of a novel virus.

Central muscle fibrosis was detected in histology of one pond (pond 5). This pathology finding was quite unusual and considered to possibly reflect either; chronic injury or trauma or previous acute muscle necrosis (e.g. due to stress) that has become fibrotic. No evidence of inclusion bodies or structures within the muscle tissue that would suggest involvement of a virus (e.g. Infectious myonecrosis virus (IMV) or Penaeus vannamei nodavirus (PVNV) infections) in this pathology. IMV and PVNV have been associated abdominal muscle fibrosis in penaeids (Lightner et al., 2004 In Naim, Brown, & Nibert, 2014;Prasad, Shyam, Banu, Jeena, & Krishnan, 2017; Tang, Pantoja, Redman, & Lightner, 2007). It is suspected that this pathology finding may reflect a chronic acute stressor (e.g. environmental conditions). When prawns are exposed to stressful conditions (e.g. low oxygen, crowding, sudden temperature or salinity drop) they can undergo muscle necrosis (Johnson, 1995). If extreme this may progress until the entire tail area takes on a whitish appearance (Johnson, 1995). If shrimp are withdrawn from the adverse environment before prolonged exposure, they may return to normal appearance (Johnson, 1995). Moderately affected shrimp, only parts of the body return to normal; other parts, typically the last segments of the tail are unable to recover and are prone to bacterial infection (Johnson, 1995). Given this finding was only detected in 17% 1 of 6 affected ponds with samples collected. It is not clear to what extent central muscle fibrosis was involved in pathology from the post-stocking mortalities, given central muscle fibrosis was only found in prawns from 17% (1/6) grow-out ponds that experienced elevated mortality and with diagnostic samples submitted.

Bacteriology and Mycology

Pathology from grow-out populations that experienced elevated mortalities during the post stocking period suggested a potential role for likely mixed *Vibrio* spp. infections. It was hypothesized that apparent increased incidence and severity of these infections were potentiated by a range of unidentified environmental stressors on the cultured larvae.

The histology findings of hepatopancreatitis with presence of gram-negative bacteria in 4% (1/25) of prawn samples suggests some similarities to the pathology observed in the hatchery. Intestinal pathology with a bacterial component was also detected in all prawns from another pond (pond 27) towards the end of the crop Also, in common with the hatchery cases, a range of potential environmental stressors (See 4.1.) were identified.

Bacterial culture performed on aseptically collected haemolymph and hepatopancreas from a grow-out pond (pond 5) that experienced elevated mortality, isolated *Vibrio* species (*Vibrio harveyi* and *Vibrio parahaemolyticus*) and *Photobacterium damselae*. The bacteria, Vibrio species (e.g. *Vibrio alginolyticus* and *Vibrio harveyi*) and *Photobacterium damselae subsp. Damselae* (also known as *Vibrio damsela*) are considered to be secondary and opportunistic pathogens (Wang & Chen, 2006; Buller, 2014), and have been demonstrated to cause disease outbreaks of vibriosis associated with poor environmental conditions (Wang & Chen, 2006), including in grow-out ponds (Sung, Hsu, Chen, Ting, & Chao, 2001; Sung, Li, Tsai, Ting, & Chao, 1999). *Penaeus monodon* suggested to a change in temperature, either increased to 34°C or decreased to 22°C from a control temperature of 26°C, resulted in greater mortalities of prawns challenged experimentally with *Photobacterium damselae subsp. damselae* than those held at 26°C without a change in temperature (Wang & Chen, 2006). Decreases in both total and differential hemocyte count and phenoloxidase activity was also identified

in these prawns, suggesting potential immune compromise following the environmental change (Wang & Chen, 2006).

The lack of significant pathology in majority of grow-out prawn samples that were collected may be due to the low sample number (discussed above) and/or the delay between occurrence of peak mortalities in affected populations and detection and sample collection. Diagnostics samples were not collected until (likely) well after mortalities had been occurring for likely some time, because reduced post stocking survival went relatively undetected until approximately 8 weeks post stocking. Dead prawns were not observed around the edge of the pond. This may result in the data outputs being more reflective of survivor animals as sample collection is influenced by a survival bias. This finding may explain why such a larger number of prawn specimens from affected pond populations were found to have no significant abnormalities detected.

Retained moult shell was detected in 23% prawns (7/31) from grow-out ponds that experienced elevated mortality and with diagnostic samples submitted. This may reflect weakness and poor normal grooming behaviour and possibly poor condition (Pers. Comms. Ian Anderson, 2019).

Haemocytic enteritis (HE) was detected in histology of one pond (pond 5). This pathology finding is less well understood. It has been suggested to be possibly caused from exposure to endotoxin released by rupturing cyanobacterial cells (Lightner 1985 In: Anderson & Owens, 2001). Anderson & Owens (2001) found an apparent, non-significant, increased relative risk (RR= 2.55) of prawns having HE when to have cyanobacteria were detected. Histopathology performed on banana prawn (*Fenneropenaeus merguiensis*) experiencing lethargy and inappetence for HE and chronic septic hepatopancreatitis (Anderson, 2005). This population had a history of rapid salinity and temperature decrease (or change) due to heavy rainfall, pond algal bloom instability, benthic algae matts and cyanobacteria developing on the pond bottom prior to onset of mortalities (Anderson, 2005). It is not clear to what extent HE was involved in pathology from the post stocking mortalities, given HE was only found in prawns from 17% (1/6) grow-out ponds that experienced elevated mortality and with diagnostic samples submitted. This pathology is more typically observed towards the end of the crop when biomass pond organics and bacteria are present in the pond environment are likely higher (Pers. Comms. Ian Anderson, 2019).

The above findings suggest it is possible there was a bacterial component of the grow-out mortalities, however a deficiency of diagnostic testing during the time mortality events were occurring may restrict the drawing of definitive conclusions.

5.5. Prospective trial pond survival and performance

5.5.1. Trial Commencement

There were no major abnormalities detected during the commencement of the trial that were considered to have negatively affected the subsequent survival and performance of the prospective trial ponds.

Water quality parameters in the transport tankers was more reflective of the hatchery water than grow-out trial pond water, which the PLs were stocked into. The transition of PLs between these two water bodies (acclimatisation) was performed over approximately 1-2 hours compared to the typical practice of 2-3 hours by the farm, due to later afternoon arrival of the PLs to the APF pond farm and heavy rainfall (>50mm) on the day of stocking. The magnitude of these water quality differences consisted of; 6.3-6.9 ppt salinity, 0.73-1.09 pH, and 2.4-2.7°C water temperature. Abrupt changes to salinity, water temperature and pH can dramatically stress prawn larvae (FAO Fisheries and Aquaculture Department, 2007). It is possible the magnitude of these the water changes during the trial pond acclimatisation, within a 1-2 hours period, exposed PLs to some physiological stress. However, this impact is thought to be minor as magnitude of water parameter change was similar in each of the three trial ponds, including trial pond 44 which had very minor mortality during the entire grow-out period. FAO Fisheries and Aquaculture Department, (2007) recommend that water parameters changes be

avoided before PL10, and that adjustments to salinity of less than 3 ppt per hour and water temperature of less than 2°C per hour. A pH change <0.5 per hour may also be helpful.

Health assessment and stress test performed at stocking did not identify any abnormalities considered likely to affect post stocking survival and performance of the PL. A large size variation in the PLs was detected however, this was apparent in all three trial ponds.

5.5.2. Trial Progression

Mortality (two dead PLs) was detected in pond 43 on day 5 of the trial. This appeared short-lived as no other mortality was observed throughout the trial. However, this may reflect a false negative result, as mortality appeared likely to have occurred during this period (see 5.5.3. and 5.6.). The use of feed trays as places for sample capture may also tend to inadvertently bias sampling towards feeding, healthier stock.

Estimated health score appeared to progressively increase over the duration of the trial, following an initial decreased, possibly due stress of pond transition. The predominant factor lowering the health score was the size variation which fluctuated marginally over time but remained evident (See also 5.5.3.).

There was a steady increase in average growth was observed in all trial pond populations from day 18 of the trial (assessment of weights and lengths commencing on day 11) (See appendix 1). No reference values from an Australian perspective could be found to compare growth rate, to determine if trial prawns were performing above or below adequate levels throughout the trial. This area requires further research work and industry data analysis.

5.5.3. Trial Outcome

Survival to harvest was estimated for each of the three trial ponds at the 8-week post stocking timepoint as per normal APF protocol. At 8 weeks post stocking estimations on pond survival to harvest were: Pond 43 = 35%, Pond 44 = 80%, Pond 45 = 35%. The prospective trial ponds where taken through to harvest with the following adjusted post-harvest estimations on pond survival of: Pond 43 = 22.83% (2065 Kg), Pond 44 = 97.80% (7572 Kg), Pond 45=29.32% (2655 Kg). All three prospective trial ponds (43, 44 and 45) did not appear to experience elevated mortality in the post-stocking period that was equivalent in magnitude to the commercial APF grow-out ponds during the 2018/19 production. The three prospective trial ponds had an estimated adjusted harvest survival greater than 74% of commercial and trial grow-out ponds (37/50) and 26% of the commercial ponds had similar mortality to the 2 trial ponds that displayed lower survival. There was an apparent harvest survival difference between each of the trial grow-out ponds, with pond 44 having survival more than three times greater than pond 43 and 45. Further investigation was performed to understand possible explanations for this survival difference (see below).

Review of predicted survival at the end of the trial (based on feed intake), differences in the average number of PLs detected on feed trays, and ultimate end harvest survival would suggest that mortality occurred during the trial period, which was not detected during the trial. It is unclear if the non-survival that occurred in the first 8 weeks post stocking (trial period) reflects a 'typical' or 'expected' outcome for this period in the Australian prawn industry or different. Significant variation is reported across industry with anecdotal reports of correlations to larval tank quality.

There was no obvious difference in health score between the ponds detected during the trial. However, interestingly the average health score was marginally greater in the higher survival pond 44 (88%), compared to the lower survival pond 43 and 45 (both 86%). This might have been due to marginal differences in size variation and hepatopancreas score over the duration of the trial. The significance of this finding is unknown given this health assessment framework has not yet been standardised or widely adopted by the industry to understand consequences of subtle differences. The difference recorded is small and may be within the error limit of this scoring system.

Not significant difference was detected between water quality parameters (average, minimum and maximum - salinity, pH, water temperature and dissolved oxygen) between all three trial ponds. Unionised ammonia (NH_3) was found at highest levels (trial average and maximum) in pond 44. Considering pond 44 had the highest estimated survival NH_3 appears not to have contributed to the lower survival in pond 43 and 45 (see appendix 1).

Very poor survival was apparent in all the pen enclosures within each of the trial ponds, which varied between each pond. The sub-set of PL housed in the small enclosures were significantly smaller than the free-ranging PL with pond 43 and 44 enclosure prawns at the end of the trial 4.1- and 2.5-times lower weight, respectively, and 2.1- and 1.7-times lower body length, respectively, than the average for the free ranging prawns in these ponds. This may be due to the significantly higher stocking density, differing water quality or feed availability. Given the success in capture of prawns from the free ranging area of the grow-out pond's further analysis of animals in these enclosures, which were by design a safeguard population, was not performed. Additional differences to the typical commercial setting were present adding further potentially confounding factors onto any conclusions drawn from survival and performance in these enclosures.

Given the success of recapture through increased feed tray deployment and observation it appears enclosures will not be required for successful early stock monitoring.

5.5.4. Molecular Samples

All three toxin genes (YaFO, RtX, and zon occludens) were detected in all three populations (pond 43, 44, and 45) at stocking into APF grow-out ponds (day 0).

There was an apparent higher average CT value in the three trial ponds compared to APF hatchery PL samples, and in trial ponds 43 and 45 compared to pond 44 (RtX and zon occludens only). This significance of these relationships is not known. Further testing of molecular samples from prospective trial ponds was not performed due to the following:

- There was no significant association detected between presence nor apparent toxin gene load (CT value) and the case definition (0% survival) in either hatchery or grow-out epidemiological analysis;

- All three toxin gene were detected in all three trial pond populations stocked into trial ponds prior to stocking, therefore these populations were not absent of the toxin genes;

- None of the three trial ponds met the case definition (0% survival), therefore presence of the toxin genes at stocking did not appear to associate with the case definition (0% survival) – aligning with the findings in the commercial ponds;

- Variation in methodology of sample collection does not allow for accurate and repeatable comparison within or between trial pond populations over the duration of the trial, nor to commercial grow-out ponds. This was due to a consistent number of animals (e.g. count) or mass of target tissue (e.g. weight) was not performed for the trial pond sample collection. Therefore, with variability in methodology we would expect to potentially influence the result output (CT value) and thereby limit the validity of greater further interpretation. Quantification of unstandardized samples is not relevant to further analysis.

5.5.5. Histopathology

Examination of histopathology samples collected during the first two weeks of the prospective trial did not detect any consistent and significant pathology that could explain survival difference between the three trial ponds, or likely to cause a significant health consequence to these prawns.

Variable levels of tissue autolysis (especially in the hepatopancreas) were apparent in majority of the preserved samples except for the smaller individuals, increasing over time in the trial as the prawns grew. Even once injecting of prawns during sampling commenced (when prawns were near ~40mm)While this restricted histological interpretation of larger prawns from latter samples of the trial, it was a very useful finding to facilitate further optimisation of fixation methods in small and growing prawns to ensure adequate preservation of the hepatopancreas and midgut, and subsequent diagnostic histopathological analysis.

The large individual prawn size variation (<70% PLs the same stage or size) that was observed in the trial pond populations, visually, was also apparent in all three trial pond populations, histologically, becoming more obvious throughout the trial as prawns grew. This is not surprising given the detection of this finding at initial stocking. As this finding was apparent in all three trial ponds, it is not clear that it caused an influence on differential trial pond results.

There were some noteworthy histology changes detected in the trial pond populations from the first two weeks of the prospective trial. Of particular interest was the bacterial proliferation on the mandibles on 2.2-9.3% of prawn's samples from all trial ponds during this period. It is unclear if this pathology has any impact on survival as pond 44 had the highest prevalence of this lesion yet was found to have an extremely high estimated survival to harvest. Pond 44 did have the lowest estimated average weight and length at the end of the trial so it may be possible this finding impacted feed intake or performance, although feed availability and stocking density effects may be alternate explanations.

The prevalence of prawns with lymphoid tissue (referring to actual lymphoid organ and, or that around the sub-gastric artery) in sections appeared to increase over time in all trial pond populations. Nakamura, (1987) observed a similar finding in *Penaeus japonicus* where there was no lymphoid organ in the younger larvae that showed similarities to the shape and position to the lymphoid organ described at the anterior region just adjacent to the mid gut gland. They found the size of the lymphoid organ increased rapidly between PL20 and PL30 (by an estimated magnitude of 3-4 times). The lymphoid organ (LO) is believed to have an important role in immune system defence against invading pathogens in penaeid prawns (Rusaini & Owens, 2010). It is unclear if the apparent increase in lymphoid tissue prevalence in the first two week of the trial relates to normal growth, increase immunological challenge, a sectioning anomaly or a combination of these.

Early spheroid formation was apparent in prawns as early as day 0 of the trial (PL20), despite not being detected in all of the samples. Similar to that described above regarding lymphoid tissue, the driver of the apparent increase spheroid formation is not clear, and could reflect normal growth, immunological challenge or suppression, increased stress, environmental factors, or increased viral loading.

Fouling of the cuticle with sessile ciliates was only found in the higher survival, pond 44, between day 6 and 8 of the trial, and not in pond 43 and 45, therefore does not appear to be associated with increased non-survival.

There is limited histology reference material available from grow-out pond prawns of this age to allow a detailed comparison to expected 'normal' tissue architecture. This project has provided some initial descriptive information which could be used to build upon, in this space, to assist future histopathological diagnostic analysis for prawns of this age class.

A review of trial pond data identified a deviation between each trial ponds was first detectable using; i) the average number of PLs observed on feed trays during health assessments (Figure 3) from day 25, and ii) the daily feed intake (Appendix 1) from day 35. These apparent differences between trial ponds remained evident throughout the trial.

The enumeration of PL's on trays may represent a more robust method for quantifying early pond survival which is reported to be notoriously difficult until approximately 6 weeks into the culture cycle. Earlier detection of low survival can permit more rapid investigation and allow farm management to consider re-stocking affected ponds to still produce a crop within the available season.

It was considered worthwhile to further examine histology samples from 25-35 days post stocking to identify potential pathology changes associated with changes in detectable prawn number and feed intake (as an indirect measure of survival). During preliminary histological examination of a sub-sample (day 32 and day 34) it was found that the level of autolysis was too significant to obtain meaningful results from this further examination. Despite injecting some of the prawns sampled there was still some autolysis evident of hepatopancreas, anterior midgut caecum and midgut.

In order to detect subtle pathology changes of hepatopancreas, anterior midgut caecum and midgut good tissue preservation is imperative. Further recommendations to optimise this technique are provided (See section 8.)

5.5.6. Additional Trial Considerations

There were several factors that differed between the commercial APF trial ponds that experienced elevated (case pond) mortality during the 2018/19 season and the prospective trials pond, which might explain the observed survival and performance difference. These factors were unable to be avoided and are considered potential confounding factors on the overall assessment.

PLs were not available from the APF hatchery for use in the trial, so PLs were purchased from an external hatchery (PRF). These PLs were of a larger size (farm comms.) and average age (PL20) than case ponds (PL12). These PLs were subject to different hatchery practices to that of APF PLs, including apparently higher survival in the hatchery (PRF hatchery not known to have experienced the mortality event that occurred at APF during the 2018 hatchery season).

The PLs used to stock the trial ponds were sourced from a different tank system (nursery tank system) and not all sourced from the same hatchery population. Pond 43 and 45 were from hatchery population N4 and pond 44 from hatchery population N3. Given there was a significant survival to harvest difference between these original hatchery populations, there may have been prior contributing factors that influenced survival, or the robustness, of these PL once in grow-out ponds. The significant size variation in the purchased PL suggest there may have been further population mixing in the hatchery prior to obtaining PLs, however these further hatchery husbandry details are not known.

Average stocking density of trial ponds (40 PL per sqm) was not able to reflect commercial pond densities (50-100 PL per sqm), which had been used earlier in the season.

Preparation of trial ponds differed from the typical commercial scenario. The previous crop had on only recently been drain harvested (30/12/19) before refilling was commenced (01/01/19) for the trial. Therefore, a typical full dry out, sludge removal, reshaping, liming and disinfection was unable to be performed before being re-filling. This may have resulted in a biological load on these ponds quite different to a typical commercial scenario. Possibly pond microbial flora were able to re-establish faster, despite shorter period between fertilisation and filling then stocking.

5.6. Post-stocking sampling methods for PL's to monitor health status

To our knowledge this is the first time a reliable method of post larvae capture from a grow-out pond has been consistently demonstrated in a commercial setting. This technique may assist farms to; perform early animal health surveillance, collect samples for monitoring pathogens, monitoring of growth and performance of animals in the post stocking period, monitoring of feed intake during the post stocking period to adjust feed approaches, and possibly early ability to predict and monitor survival in the post stocking period.

For example, using the current results if using the adjusted end harvest survival of the highest survival pond, pond 44, was used as a benchmark (97.8%), then a calculated survival predication for pond 45 and 43 would have be 46.2% and 26.7%, respectively. While this prediction differed slightly from ‘actual’ end harvest survival in these ponds of 29.3% and 22.8%, for pond 45 and 43, respectively,

this assessment could be made significantly earlier (at 3.5 weeks vs 8 weeks) and with more apparent accuracy (3.8 - 8.3% closer) than the current method of estimating survival using feed intake at 6-8 weeks post stocking (APF farm Comms.). Further replication of these finding is needed to build confidence in the use of this monitoring as survival predictor tool.

Due to a historical inability to recapture recently stocked larvae there is very little industry data on the 'normal' histology from *Penaeus monodon* larvae during the post stocking period in an Australian commercial setting. This study has potentially refuted industry acceptance that sampling PLs during this period is not possible, which allows for further research into animal health during this period, including optimal preservations techniques (See 5.5.5.) and recording 'normal' growth and performance during this stage of production to shape and design industry or farm benchmarks.

The use of feed trays to sample post stocked PLs may select more preferentially for appetent (feeding) and healthy animals over inappetent (non-feeding) and unhealthy animals, potentially generating a selection bias on the prawns captured for diagnostic testing. This was unavoidable given there were no alternative capture methods that have been proven effective to sample post stocked free ranging PLs from an earthen pond. Some additional ideas were considered (including a fine mesh sled or plankton tow nets), however these or other designs were not trialled given the success of the feed trays to capture free ranging PLs. Lavilla-Pitogo et al., (1998) also showed the capture of post stocking PLs was possible

Additionally, prawns were sampled from the edge of the pond, potentially generating another selection bias on the prawns captured for diagnostic testing. Sampling fish from the periphery or edge of a population can result in an increased risk of capturing smaller and more unhealthy individual animals (Fensham, Bubner, D'Antignana, Landos, & Caraguel, 2018). Little information is not known about the health status and performance of free ranging prawns in an earthen grow-out pond in relation to their distribution within the pond, however it would be expected to be non-homogeneous.

Necessary replication of these techniques in more commercial pond populations is required, alongside comparison with commercial farm data to confirm this finding and provide necessary confidence in wider implementation of this farm technique. Significant benefits may exist if widespread implementation assists improved ability to detect and make a management decision regarding when early crop mortality has occurred in a pond and/or to trigger and facilitate further health investigations

5.7. Levels of agri-chemical pollutants in source water entering ponds and potential impact on prawn larval health

The survival to harvest of trial pond 44, which containing the replicate empore disk passive water samplers, was very high, at and adjusted estimate of 97.80%. This was significantly higher than the adjacent grow-out trial ponds 43 and 45, which had an estimated adjusted survival to harvest of 22.83% and 29.32%, respectively. Therefore, the time weighted average (TWA) concentration and species of PPCPs that were detected, in this scenario, did not appear to cause direct harm to the cultured prawns (*Penaeus monodon*). It is assumed that the concentration and type of PPCPs and neonicotinoid chemical compounds that prawns in adjacent grow-out trial ponds 43 and 45 would be similar to what was detected in pond 44, as pond filling and water flow management was largely similar. However, some differences could not be ruled out. It is not known whether the concentration and species of PPCPs and neonicotinoid chemical compounds differed over time between the stocking period of the commercial grow-out ponds and the trial grow-out ponds at APF. The detected chemicals compound were predominantly herbicides used in the agriculture industry to control weeds.

Recent water contaminant monitoring on Australian prawn farm source water performed by Hook et al. (2018) identified a number of similar herbicides, as were detected in this project. 2,4-dichlorophenoxyacetic acid (2,4 D), atrazine, diuron, hexazinone, MCPA (2-methyl-4-chlorophenoxyacetic acid) and tebuthiuron were found in both this research projects. The study by Hook et al. (2018) detected 2,4-dichlorophenoxyacetic acid (2,4 D), atrazine, diuron, hexazinone and MCPA (2-methyl-4-chlorophenoxyacetic acid) in Mackay region prawn farm source water, as we

detected in the APF trial ponds, while this project also detected tebuthiuron. These chemicals were detected at apparently higher concentrations by Hook et al. (2018). However sample locations differed with Hook et al. (2018) who targeted river/estuary locations used by farms, whereas in this project we targeted the production ponds containing the prawns. Detected TWA concentration of diuron, hexazinone, and tebuthiuron were below proposed marine aquatic ecosystem protection guidelines for the GBR catchment (King, Smith, Mann, & Warne, 2017). The Hook et al. (2018) study did not test for bromoxynil, nor the chemical compound metabolites of ametryn hydroxy and desethylatrazine, which were found in this project.

Some of the above chemical compounds detected in this project are no longer permitted for use as active substances in herbicide products overseas due to their toxicity to aquatic life and persistence in the environment, despite remaining registered and permitted for use in Australia. These include: tebuthiuron (King et al., 2017), and atrazine (Sass & Colangelo, 2006), which do not have regulatory approval for use in Europe. Diuron, atrazine, hexazinone, tebuthiuron and ametryn hydroxy have been highlighted as priority herbicides by management agencies as part of the overall Great Barrier Reef (GBR) management and protection plan (Mercurio et al., 2016). See King, Smith, Mann, & Warne, (2017) for further information ecotoxicological information regarding diuron, hexazinone, and tebuthiuron.

No levels of individual compounds were above levels that were considered to cause adverse effects in aquatic ecosystems. The effect of the mixture of compounds is not known but may exceed the impact of the compounds when assessed on an individual basis (Chen, Wang, Qian, Zhao, & Wang, 2015; Xiao et al., 2018). The performance of the examined trial pond with high survival aligns well with this result, where an adverse effect from measured agrichemical exposure would not be anticipated. Due to the, at times, pulsatile movement of agrichemicals into water supplies in relation to application timing and rainfall (Mercurio et al., 2016), the role of agrichemicals in the previous events cannot be determined. Some literature suggests that chemical Australia risk assessments which underlie approved uses are inappropriate to prevent aquatic toxicity (Holmes, 2014). So while this project did not identify any contaminants at concern levels and/or an association with negative impact on prawn survival, water contaminants may remain a potential risk factor to contribute to negative prawn and pond ecosystem health, where they continue to enter prawn farms in variable concentrations and mixtures.

5.8. Further development

A single consistent infectious organism (virus, bacteria, fungi, or parasite) was not identified as a primary driver of the mortality in either the hatchery or grow-out stage of production either through the prospective trial or epidemiological data analysis.

A range of potential risk factors were identified with differing strength of associated with mortality in the hatchery and grow-out stage of production (see sections above). These factors can be used as an evidence base to direct changes to current farm practices and protocols to reduce the associated risk of future mortality (See section 8.).

Further review of data collected from the upcoming production season may assist strengthening or weakening of the significance and probability of the identified risk factors. In addition, where new issues are identified in the hatchery or grow-out stage an investigative framework has been trialled to allow for improvements to sample collection, processing, analysis and results interpretation.

6. Conclusion

There were no new, emerging or historically described infectious organisms identified in this project as primary pathogens that could fully explain the observed mortality. A bacterial component was identified with the isolation of bacteria (e.g. *Vibrio* sp.), which are considered ubiquitous in the marine environment, and potential opportunistic pathogens, especially in larvae. The ability to completely avoid and eliminate these bacteria from exposure to prawn culture is near impossible, and likely not desirable as the prawns need to establish microbiomes. While it is imperative to reduce exposure of prawn larvae to these potentially harmful bacteria in the hatchery, despite all the water disinfection (filtration, ozone, UV, etc) it is a common observation that bacterial load is typically found to increase during the larval production (Sathish Kumar et al., 2017; Kumara & Hettiarachchi, 2016). Avoidance of these bacteria in hatchery tanks and earthen grow-out ponds is unlikely to be achievable dictating that they be managed during all stages of production.

This project utilised NGS to identify genetic material from potentially harmful toxins (YaFO, RtX, and zon occludens). This allowed for the development of novel diagnostic qPCR tests for these toxin genes to assess their potential involvement in the observed hatchery and grow-out mortalities. This in turn allowed exploration of the JCU Aquapath hypothesis of a role for bacterial toxins in the mortality events.

The prospective trial ponds did not encounter any instances of zero survival. Two ponds experienced low survival and one pond experienced very high survival. Useful methods to capture and preserve prawns following stocking into an earthen grow-out pond environment were trialled successfully. Further work is required to fully optimise these techniques that offer benefits through monitoring early survival of PLs.

The use of retrospective epidemiological analysis used an evidence-based approach to assess identified potential risk factors to shape the project recommendations. The toxin genes (YaFO, RtX, and zon occludens) did not appear to significantly associate with the mortality observed in the hatchery or grow-out farm within the limits of the available data and analysis undertaken. What stood out was a range of risk factors that were associated with disease, which likely through co-contributing, each in part, potentially creating prawns which were less able to withstand opportunistic infection from marine microbes. While there was no single predominant marine microbe identified in the mortality investigation, this could be due to insufficient additional bacterial cultures performed at the time of the mortality outbreak.

The extent of the retrospective epidemiological analysis was limited by the available data set. In addition, a lack of further defining characteristics of mortality events encountered in the hatchery and grow-out stages limited the epidemiological case definition to mortality alone (defined at culled population, 0% survival). In the grow-out stage, data was only available from 47 populations, which may have limited the significant risk factors that were able to be identified in the analysis.

A holistic approach allows a broad range of contributing factors to be identified. Through addressing these factors it is anticipated that improvements to overall prawn health and survival will be achieved through generating conditions that support a more robust and resilient animal. The overall project findings broadly aligned with field investigations performed during the mortality events.

7. Implications

This project demonstrated the use of a disease investigation framework in an Australian prawn farm. This approach can be replicated throughout the industry, to assist future disease occurrences. We demonstrated how NGS can be used to identify potential pathogens not readily detectable by common diagnostic modalities, and to develop new diagnostic tests, which may have wider use for the industry.

The use of epidemiology data analysis to shape evidence-based recommendations to change hatchery and grow-out farm practices may yield benefits with wider industry adoption and provide valuable information to consider when designing or re-designing prawn hatcheries and ponds farms.

We disproved the industry acceptance that it was not possible to reliably capture young post larvae following stocking into an earthen grow-out pond. Further work is required to optimise this technique, possibly offering benefits for earlier survival prediction and better monitoring growth, performance and survival in the immediate post stocking period. These may have significant production benefits to industry with further validation and data generation.

We provided further guidance to optimise performing health assessments on post larvae, including sample collection and processing for molecular biology, microbiology and histopathology. Consideration of the proposed approaches will improve the quality and value of the testing outputs.

8. Recommendations

8.1. Hatchery

1. Avoid merging hatchery tanks that have experienced some loss or mortality.
2. Stock hatchery tanks at a density of less than 900,000 nauplii (<112 PL/L).
3. Consult with your farm veterinarian to assist with supply and strategic use of oxytetracycline hydrochloride during larval rearing (in addition to further investigation to better understand the contributing drivers of mortality) where use is indicated.
4. Consult with your farm veterinarian to assist with supply and strategic use of formalin during larval rearing (in addition to further investigation to better understand the contributing drivers of mortality), where use is indicated.
5. Review of the hatchery facility biosecurity to improve the separation of adjacent rearing room and subsequent run transmission of potential pathogens.
    - Compartmentalisation creates options for better tank dry-out and sanitary protocols.
6. Perform routine monitoring of tank water quality (water temperature, pH, DO, salinity, TAN, NH_3-calculated) to ensure optimal conditions are being maintained (e.g. water temperature (28–32C), salinity (30-35 ppt, until PL10-12 onwards), pH (7.8–8.2), unionised ammonia (<0.1 mg/L).
    - Where sub-optimal water quality is detected ensure prompt action is taken (e.g. water exchange) to avoid exposing larvae to sub-optimal environmental conditions (e.g. elevated salinity, elevated pH, elevated unionised ammonia).
    - Consider use of biological filtration on larval rearing tanks to reduce risk of excess NH_3.
7. Perform routine health assessments on all hatchery tanks using gross examination and microscopy (See project methods above for guidance and farm veterinarian for training if required).
8. Perform routine monitoring of bacterial loads in larval rearing tank inputs (incoming water and live feeds).
9. Increase the frequency of all feed inputs (algae, artemia, artificial) to ensure each hatchery tank has feed provided at least every 4 hours (24 hours per day).
    - Perform routine monitoring of feed concentrations in larval rearing tanks to adjust subsequent additions (e.g. prior to the following addition) and maintain appropriate level of each feed type available to avoid under and over feeding.
    - Perform routine inspection of feed intake from cultured larvae (see Section 8.1.8)
10. Perform gentle filtering and rinsing the live algae with clean disinfected system water using a fine-mesh bag to concentrate the algae prior to addition to larval rearing tanks to reduce the total water content of this addition.
11. Use only fresh newly hatchery live or blanched artemia. Avoid storage of this product as risk of bacterial contamination is moderate and nutritional value may be reduced with storage.
    - Disinfect live feeds prior to feeding.
12. Consider feasibility of investment in increased desalination capacity to allow improved ability to maintain optimal salinity and pH in the hatchery system with higher exchange rates.

General recommendations of relevance to hatchery management

1. Continue to monitor ciliate parasite levels in larval rearing tanks to gather further data to determine if control is warranted.
2. Monitor and minimise organic load in tanks and overfeeding.
3. Increasing water exchange and improving water quality throughout larval rearing.
4. Collection routine sample of each hatchery tank population prior to stocking into grow-out ponds (e.g. histopathology and molecular biology), alongside performing a health assessment (see Section 8.1.8).
    - Include monitoring of average PL length and weight.
5. Slowly adjust the water parameters of larval rearing tanks (pH, water temperature, salinity) to those of destination growout ponds gradually over 1-2 days immediately prior to harvest to minimise risk of physiological stress to PLs during acclimatisation and stocking into grow-out ponds.
6. If elevated mortalities are encountered perform a thorough detailed health investigation. Consult your farm veterinarian for advice.

Clinical trials to consider in future that may alter larval tank rearing conditions

- Further investigate the plastic tank coverings through a trial to determine if reduced light penetration can improve water quality fluctuations identified in hatchery tanks.

Further research opportunities.

- Consider further research into industry wide antibiotic minimum inhibitory concentrations (MIC) for commonly isolated bacteria in prawn hatcheries to optimise treatment protocols.
- Consider further research to identify the potential beneficial pathway through which erythromycin and oxytetracycline may improve larval tank survival.

8.2. Grow-out

1. Extend the minimum period between pond preparation (fill and fertilisation) and stocking PL. Ensure ponds are prepared early (e.g. minimum 2 weeks) to allow water quality and pond conditions to stabilise prior to stocking.
2. Avoid exposure of prawns to low (<36 ppt) and large fluctuations (>6ppt) in salinity during the post stocking period (5-6 weeks).
    - Consider adjustments depending on source water salinity, such as: timing of pumping, volume of pumping, volume of pond water exchange.
    - Consider investment into expansion of post pumping incoming water settlement and storage to increase capacity to exchange water during periods of sub-optimal environmental conditions (e.g. elevated rainfall reducing incoming water salinity and quality). This may also allow for additional water treatment before use (e.g. filtration, disinfection) that can offer biosecurity benefits in preventing pathogen incursion.
    - Consideration of hypersaline storage may assist in moderating rainfall effects.

3. Continue to monitor pond algae species and abundance.
    - Consider action when any low levels of Nitzschia sp. or Gymnodinium sp. are detected in the post stocking period (e.g. increased water exchange, algicide chemical addition, adjustments to feed inputs, addition or fertilisers or chemicals to shift the algae species assemblage).
4. Stock grow out ponds with PL of an age greater than PL10
    - General guideline practice suggest stocking PL15-20, unless first stocking into a nursery system.
5. Perform routine monitoring of pond water quality (water temperature, pH, DO, salinity, TAN, NH_3-calculated) to ensure optimal conditions are being maintained (e.g. water temperature (28–32C), salinity (30-35 ppt), pH (7.8–8.2), unionised ammonia (<0.1 mg/L).
    - Where sub-optimal water quality is detected ensure prompt action is taken (e.g. increasing or decreasing water exchange, appropriate product addition – fertiliser, lime, molasses) to avoid exposing larvae to sub-optimal environmental conditions (e.g. elevated salinity, elevated pH, elevated unionised ammonia).
6. Perform routine health assessments on all grow-out ponds using gross examination (Including average length and weights), microscopy (see project methods above for guidance), sample collection (histopathology and molecular biology), at least every 1-2 weeks during the post stocking period (see methods for more guidance).
    - Use additional feed trays (minimum 3 feed trays per pond), ensuring they are kept clean and maintained (at least every 2-3 days), to assist capture of prawns during the post stocking period. Ensure feed trays are first placed adjacent to where PLs are stocked in the pond.
    - Compare commercial grow-out performance and survival data to health assessment and further diagnostics data during the post stocking period and throughout grow-out to harvest to allow for earlier intervention and management actions on these populations throughout the post stocking period (e.g. trigger health investigations, population culling, pond management changes). Further data is required to validate this approach.
7. For fixation of samples for histology - use chilled (4°C) Davidsons solution (Kasornchandra, 1998) as the preferred fixative of Penaeus monodon larvae once they develop a hard cuticle. Any prawn of a total length 25mm or more need have copious Davidsons solution injected throughout the entire cephalothorax, and throughout the abdomen once a total length 50mm or more, to achieve adequate fixation of internal structures. A 1mL insulin syringe can be used to inject fixative. The prawns must be injected as soon as they have been removed from the pond, preferably pond-side, however, if not possible they should be taken from the pond and put immediately in a bucket of pond water with aeration to be kept alive, for a brief period only, until injected with Davidsons solution and processed. During this holding process (if performed) equivalent water quality parameters to the pond environment should be maintained (e.g. temperature, dissolved oxygen, pH, salinity, ammonia, stocking density). As the prawns increase in size (e.g. above 50mm) multiple injections may be required in the cephalothorax to adequately fix the entire hepatopancreas and intestine. Any prawn of a total length 100mm or more can to have the cephalothorax dorsal (top) and ventral (bottom) cuticle cut, along the length of the prawn, to improved fixative penetration. Injected prawns need to be immersed in Davidson's solution at a ratio of 1part prawn tissue to 10 parts Davidson's solution, then placed into the fridge at 4°C for optimum preservation. Use multiple or larger containers if needed but avoid reducing the fixative: tissue ratio. Keep prawn samples within Davidsons solution in the fridge for 48 hours (24 hours minimum), before transfer to 50-70% ethanol. Even the best process will not result in 100% of the prawns being perfectly preserved, so always sample 10% more prawns than you need, to ensure there are sufficient samples to for histology. It is valuable to demonstrate the best approach to properly preserve prawns for histology and this has been done

in the past at different workshops. As new technicians come into the industry these basic methods need to be redemonstrated.

- Ensure all staff making up, storing or working with Davidsons solution follow relevant MSDS and WH&S guidelines.

8. For fixation of samples for routine molecular biology – use a standardised methodology for capture and sampling of PLs for molecular biology testing (e.g. counting 100 PLs) to allow for improved ability to compare test outputs between different populations and within the same population over time.

General recommendations of relevance to grow-out pond management

1. Avoid exposure of prawns to large fluctuations in pH, DO and water temperature in the post stocking period.
2. Avoid stocking PLs into grow-out ponds over an extended number of days (greater than 1 week).
3. Consider investment in a nursery facility or system to allow for larger and older PLs to be produced prior to stocking into grow-out ponds.

8.3. General

1. Based on the data within this project consider reducing number of population extraction replicates tested from 5 to 2 for any future qPCR testing of YaFO, RtX, and zon occludens toxin genes that is performed.
   - Consider further repeatability and diagnostic validity assessment of other molecular biology testing to optimise the methodology and ensure best value for information output.
   - Additional test validation is warranted to better understand effects of pooling, homogenisation on test Sensitivity and specificity.
2. Standardise the methodology for capture and sampling of PLs (and adult prawns) for molecular biology testing (e.g. counting 100 PLs) to facilitate relevant quantification of all data outputs.
3. Where possible, standardise the molecular biology processing methodology to assist veterinary interpretation of test results.
4. Utilise the services of a registered veterinarian for assisting disease investigations and diagnostic interpretation of all prawn populations.
5. Consider using passive water samplers around the time of stocking as a sensitive method of assessing agrichemicals that may enter prawn farms. Further research is required to better understand the agrichemicals risk profile and potential impacts.
6. When allocating animals into a trial ponds ensure that a systematic randomised allocation approach is used to reduce the risk of introducing bias into the treatment recruitment.

9. Extension and Adoption

The findings of the field and laboratory investigations are collated in this report, which will be available online.

This information will help guide a further internal review of APF procedures to improve future hatchery and pond production outcomes.

The findings of the project will be reported at the Annual Australian Prawn Farmers Symposium 2020 and shared to members of the Australian Prawn Farmers Association through industry-based conferences/workshops.

FFVS core consultancy services include veterinary mortality investigation. This report demonstrates the potential outputs from such activities and will encourage other farms within the industry to adopt this approach to assist similar reviews of current industry procedures to seek improvements to currently unexplained mortality events that are known to occur within the industry and impede production expansion.

Australian Prawn Farms (APF) may elect to keep some aspects of historical commercial management in confidence. APF will remain the data custodian at the conclusion of the project.

9.1. Project coverage

Not applicable

10. Glossary

Linear Regression - is a linear approach to modelling the relationship between a scalar response (or dependent variable) and one or more explanatory variables (or independent variables).

Logistic Regression – is a statistical model that uses a logistic function to model a binary dependent variable.

Mixed effects logistic regression - is complex statistical model that is used to model binary outcome variables, in which the log odds of the outcomes are modelled as a linear combination of the predictor variables when data are clustered or there are both fixed and random effects.

Collinearity - is a condition in which some of the independent variables are highly correlated.

Odd ratio (OR) – is a statistic that quantifies the strength of the association between the exposure and the outcome. If the OR is equivalent to 1, then the odds of the outcome are the same in either the presence or absence exposure. If the OR is below 1, then the odds of the outcome is negatively correlated with the exposure (e.g. the exposure reduces the odds of the outcome). If the OR is above 1, then the odds of the outcome positively correlate with the exposure (e.g. the exposure increases the odds of the outcome).

Spatial – relating to space.

Temporal - relating to time.

11. Project materials developed

Toxin gene qPCR (YaFO, RtX, zon occludens) diagnostic test.

Method for early recapture of post-larvae in production prawn ponds

Method for preservation of various age prawns for histology.

Framework for disease investigation on an Australian prawn farm.

Australian Prawn Farms (APF) hatchery and grow-out summary data set and statistical software (STATA) coding (available upon request).

Appendix 1: Hatchery, Grow-out and Prospective Trial Pond Figures and Tables

Table 14. Average survival in larval rearing and percentage of culled tanks in each rearing room and hatchery run during the APF 2018 hatchery season.

Run	*Rearing Room*	*Average Larval Rearing Survival (%)*	*Tanks Culled (%)*	*Tanks Culled (#)*	*Total Tanks (#)*
1	1	4.55%	63.64%	14	22
	2	16.38%	27.27%	6	22
	3	12.07%	50.00%	12	24
	Total	**11.00%**	**47.06%**	**32**	**68**
2	1	0.00%	100.00%	22	22
	2	0.00%	100.00%	22	22
	3	0.00%	100.00%	24	24
	Total	**0.00%**	**100.00%**	**68**	**68**
3	1	0.00%	100.00%	20	20
	2	2.54%	66.67%	14	21
	3	30.63%	12.50%	3	24
	Total	**11.06%**	**56.92%**	**37**	**65**
4	1	34.91%	13.64%	3	22
	2	35.29%	0.00%	0	22
	3	19.21%	0.00%	0	15
	Total	**29.81%**	**4.55%**	**3**	**59**

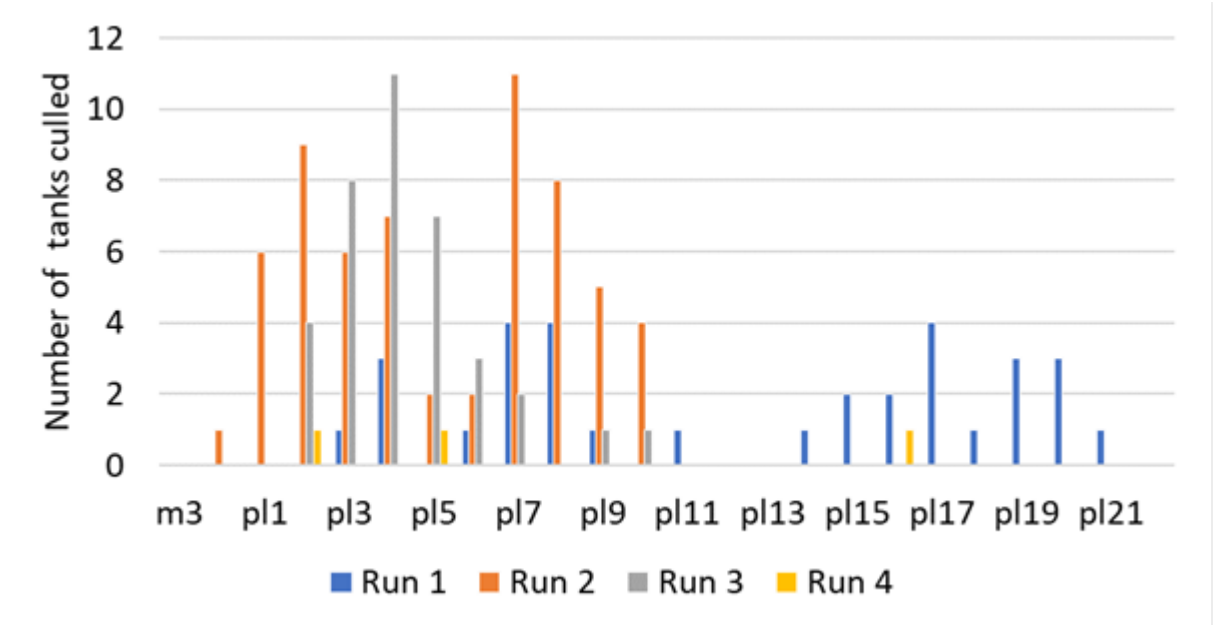

Figure 5. Daily number of hatchery tanks culled during the 2018 APF hatchery season due to detection of high mortality, during each hatchery run, over time since stocking.

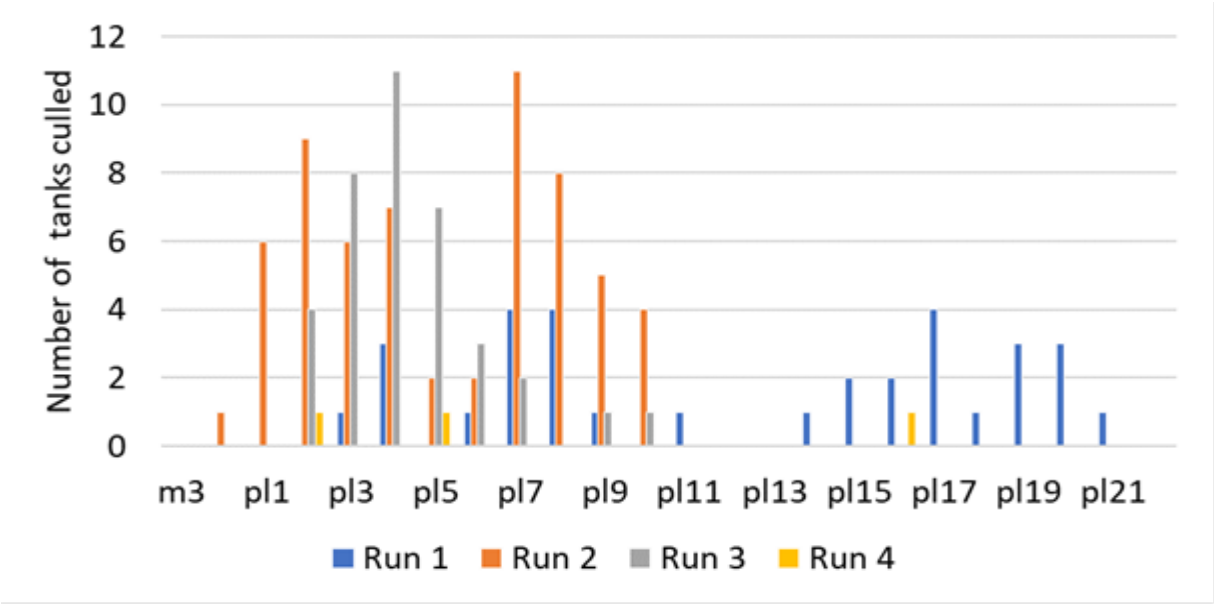

Figure 6. Daily number of hatchery tanks culled during the 2018 APF hatchery season due to detection of high mortality, during each hatchery run, over each developmental stage (age class)

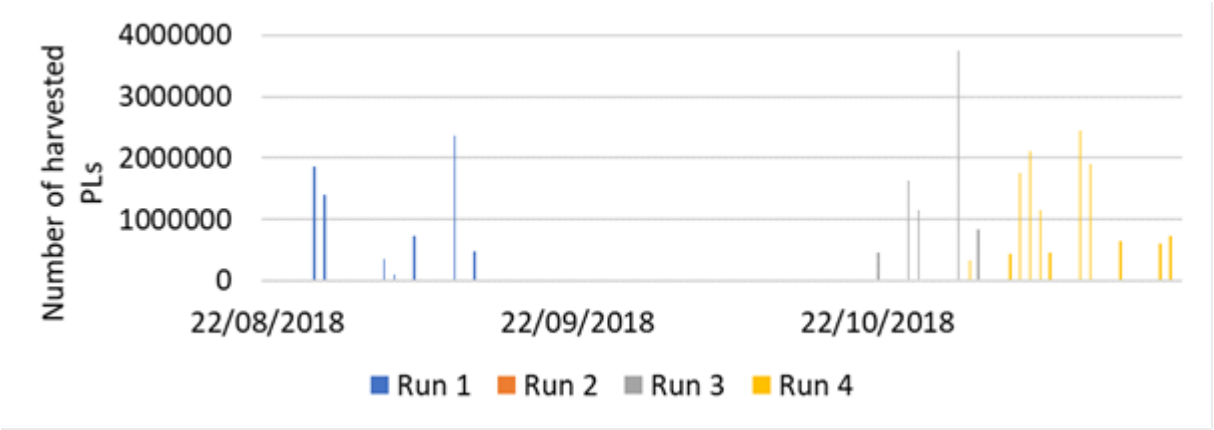

Figure 7. Number of PLs harvested from tanks, during each hatchery run, over time since stocking from the 2018 APF hatchery season.

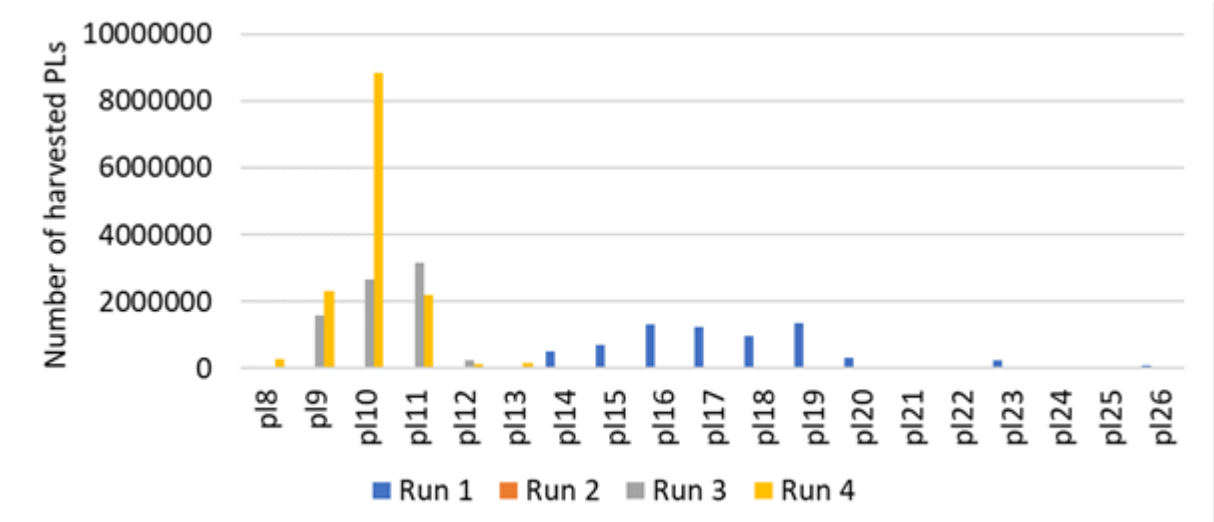

Figure 8. Number of PL harvested from tanks, during each hatchery run, over each developmental stage (age class) from the 2018 APF hatchery season.

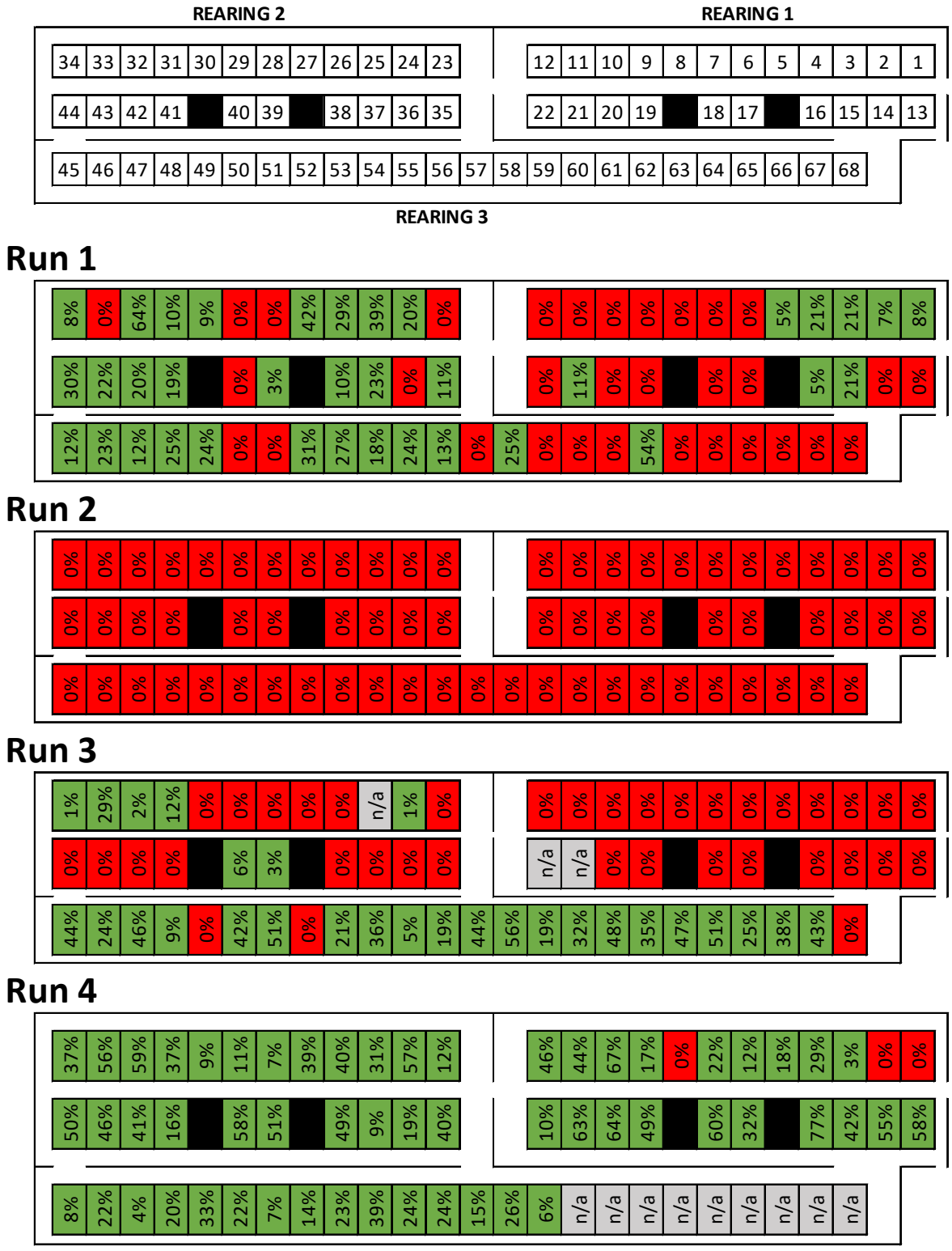

Figure 9. Spatial Distribution of mortality (as a percentage survival) in each run, rearing room and hatchery tank from Run 1-4. Colour reflects survival 0% (red = case tank), >0% (green = non-case tank). no data (grey), no tank (black). Tank numbers and rearing room details shown in the top schematic.

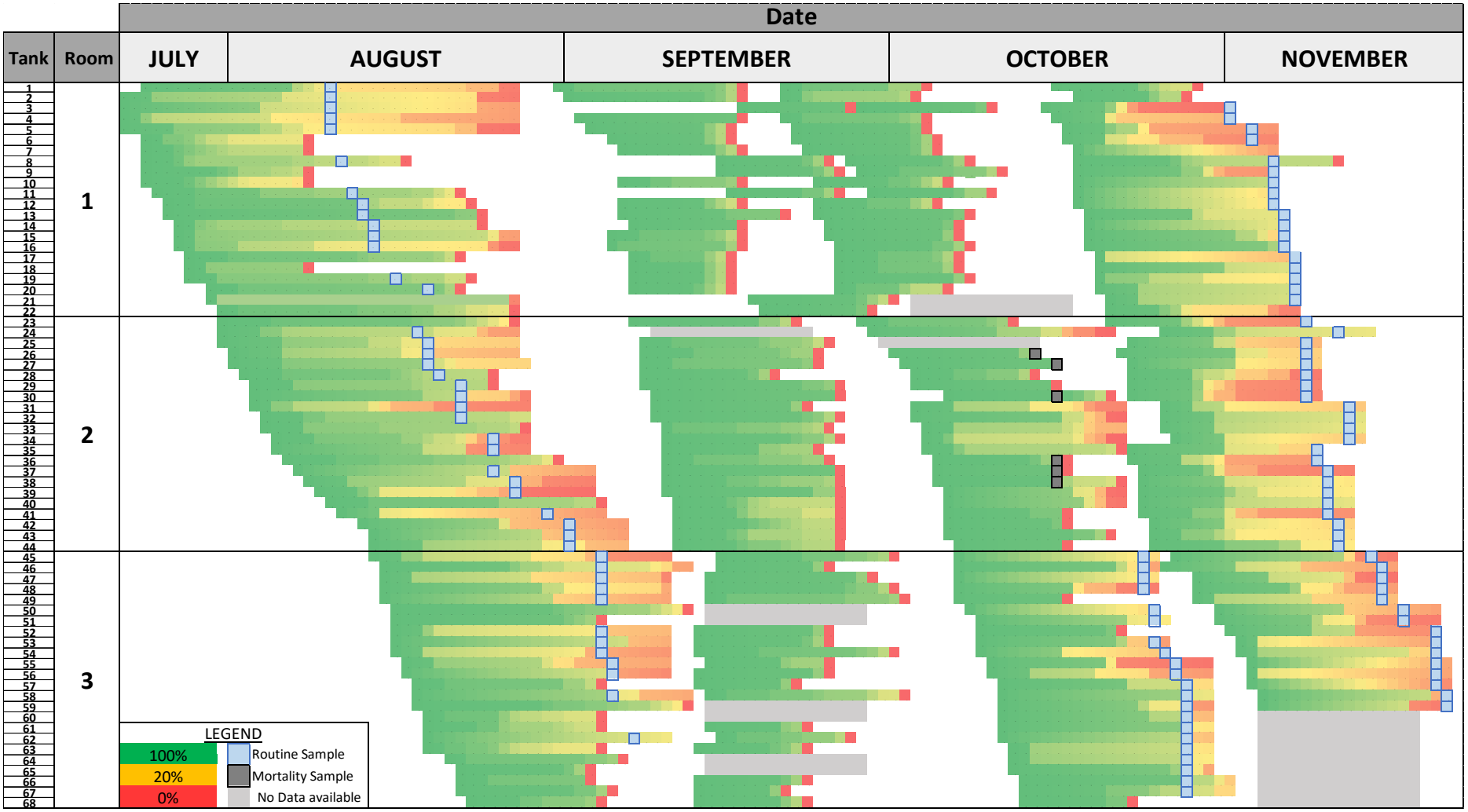

Figure 10. Spatial and Temporal distribution map displaying stocking sequence and estimated percentage of population survival throughout larval rearing. Colour chart indicated estimated percentage survival of each tank during larval rearing (100% = green; 20% = yellow; 0% = red). Blue border boxes reflect routine sampling of PL from populations for further testing and Black border boxes reflect ad hoc sampling of PL from populations experiencing elevated mortality. White colour reflect day when no stock is within larval rearing tanks and Grey when no information was available.

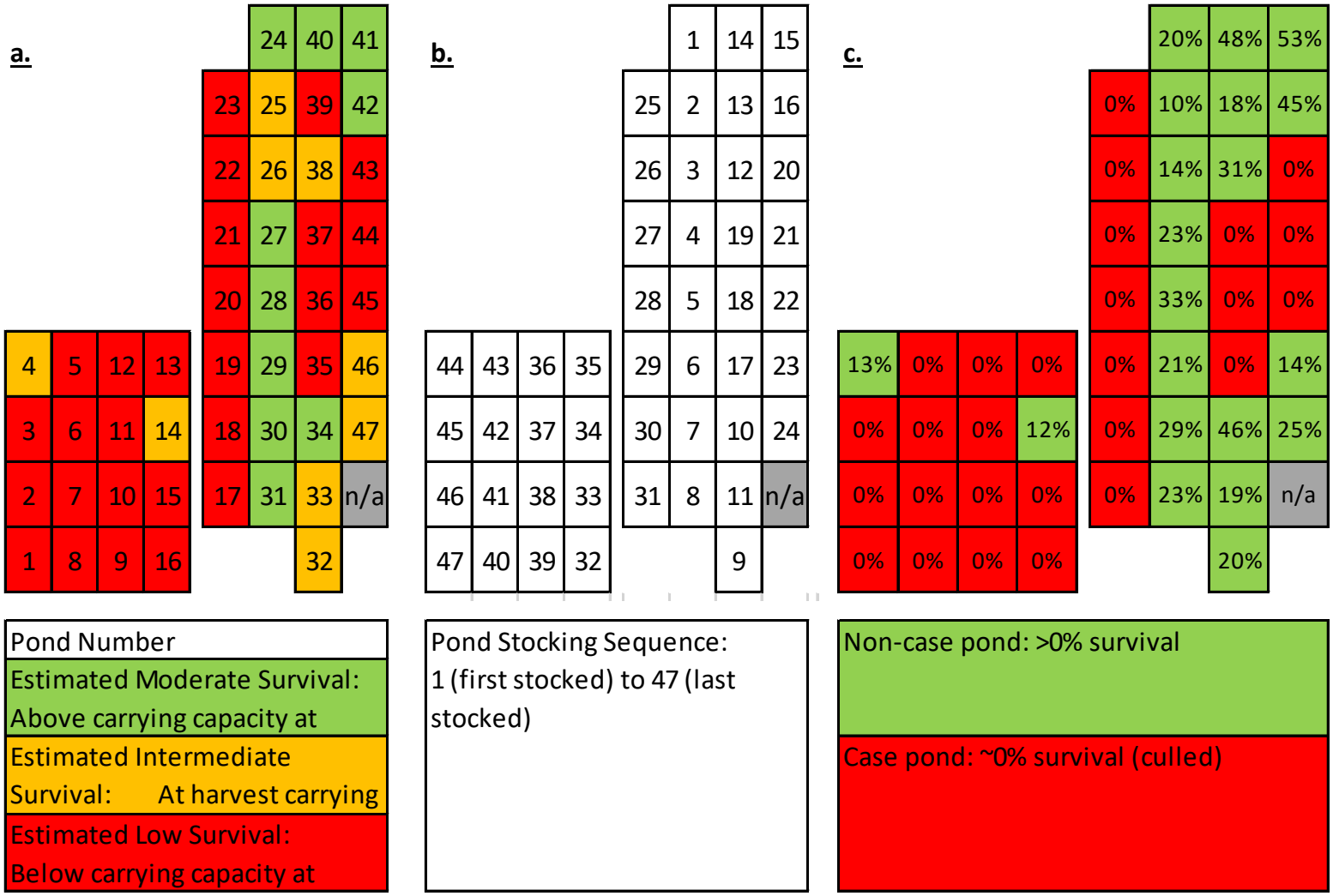

Figure 11. Spatial Distribution of mortality as estimated by a. grow-out farm stock assessment 8 weeks post stocking; b. pond stocking sequence; c. adjusted harvest survival (percentage); Legends provide further details about colour codes.

Table 15. 2019 APF Grow-out pond production details (date, PL age, Number, hatchery survival, hatchery run, pond preparation) and survival information (percentage and date).

Pond ID	Stocking Start Date	Average PL Age	Number of PLs	Average combined Hatchery Survival (%)	Hatchery Run	Days between pond fill and stocking	Adjusted Estimated Harvest Survival	Harvest Date
1	20/11/2018	11	455975	17%	4	10	0%	3/01/2019
2	20/11/2018	11	450000	28%	4	10	0%	4/01/2019
3	16/11/2018	10	450000	20%	4	6	0%	3/01/2019
4	13/11/2018	10	532591	23%	4	3	13%	7/05/2019
5	13/11/2018	10	428393	25%	4	6	0%	10/01/2019
6	13/11/2018	10	424491	26%	4	5	0%	10/01/2019
7	13/11/2018	10	432591	32%	4	6	0%	9/01/2019
8	13/11/2018	11	432591	25%	4	5	0%	8/01/2019
9	12/11/2018	10	432591	28%	4	6	0%	5/01/2019
10	12/11/2018	10	432591	26%	4	6	0%	7/01/2019
11	12/11/2018	11	432591	27%	4	7	0%	7/01/2019
12	12/11/2018	11	432591	30%	4	7	0%	8/01/2019
13	12/11/2018	11	432591	28%	4	9	0%	9/01/2019
14	9/11/2018	11	432591	21%	4	5	12%	7/05/2019
15	9/11/2018	9	432591	22%	4	5	0%	12/01/2019
16	8/11/2018	9	438301	17%	4	4	0%	11/01/2019
17	8/11/2018	10	600000	37%	4	10	0%	19/12/2018
18	7/11/2018	9	600000	28%	4	9	0%	20/12/2018
19	7/11/2018	10	600000	63%	4	9	0%	21/12/2018
20	7/11/2018	10	600852	54%	4	9	0%	21/12/2018
21	6/11/2018	10	587581	56%	4	9	0%	21/12/2018
22	6/11/2018	10	600000	60%	4	9	0%	20/12/2018
23	6/11/2018	10	600000	49%	4	9	0%	21/12/2018
24	27/08/2018	19	792830	30%	1	16	20%	7/03/2019
25	27/08/2018	25	1000000	16%	1	16	10%	19/03/2019
26	27/08/2018	18	1000000	25%	1	16	14%	21/03/2019
27	28/08/2018	17	986286	23%	1	17	23%	5/03/2019
28	3/09/2018	16	879833	19%	1	23	33%	26/03/2019
29	10/09/2018	15	1000000	27%	1	27	21%	8/04/2019
30	10/09/2018	17	1005970	25%	1	27	29%	9/04/2019
31	10/09/2018	19	834085	20%	1	27	23%	10/04/2019
32	22/10/2018	10	463607	8%	3	24	20%	3/04/2019
33	25/10/2018	11	500000	34%	3	27	19%	11/04/2019
34	25/10/2018	10	758523	38%	3	27	46%	15/04/2019
35	30/10/2018	11	800000	44%	3	5	0%	20/12/2018
36	30/10/2018	11	802648	38%	3	5	0%	20/12/2018
37	30/10/2018	10	600000	41%	3	3	0%	20/12/2018
38	25/10/2018	10	502741	32%	3	26	31%	14/03/2019
39	25/10/2018	9	540399	47%	3	26	18%	27/03/2019
40	26/10/2018	9	595542	36%	3	27	48%	16/04/2019
41	30/10/2018	10	610281	49%	3	23	53%	17/04/2019
42	30/10/2018	11	600000	34%	3	23	45%	29/04/2019
43	1/11/2018	10	607422	37%	3&4	7	0%	30/12/2018
44	1/11/2018	10	600000	17%	4	7	0%	30/12/2018
45	5/11/2018	10	600000	32%	4	12	0%	30/12/2018
46	5/11/2018	10	600000	53%	4	9	14%	28/03/2019
47	5/11/2018	10	600000	53%	4	9	25%	4/04/2019

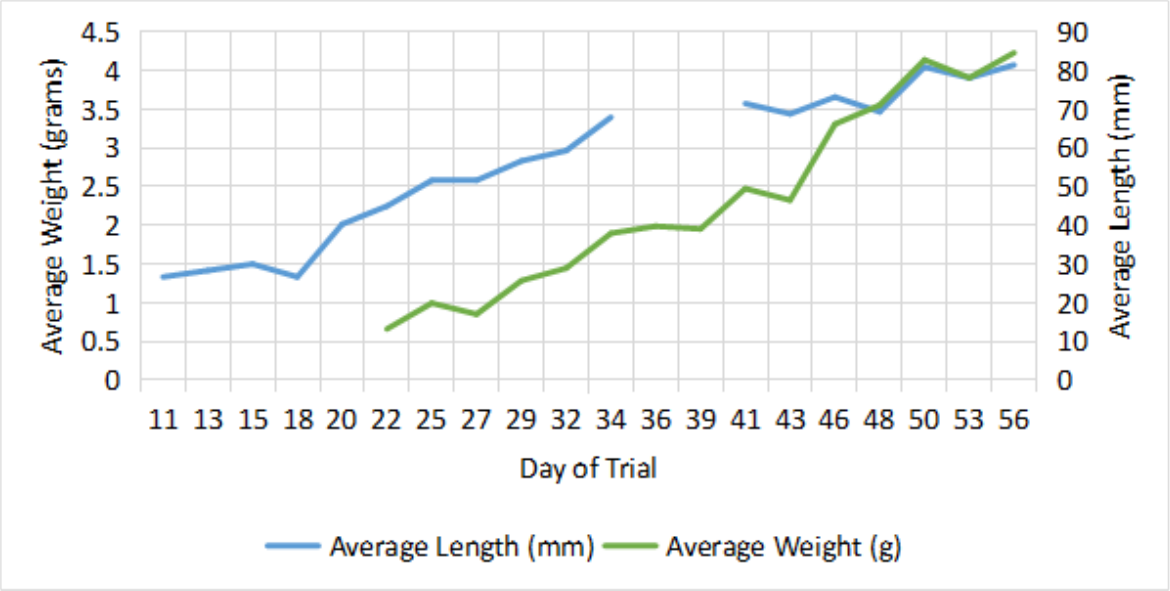

Figure 12. Comparison the growth characteristics, estimated average weight (grams) and length (mm) of PL 20 stocked P. monodon from day 11-day 56 of the trial. Note length data missing from day 36 and 39 of the trial.

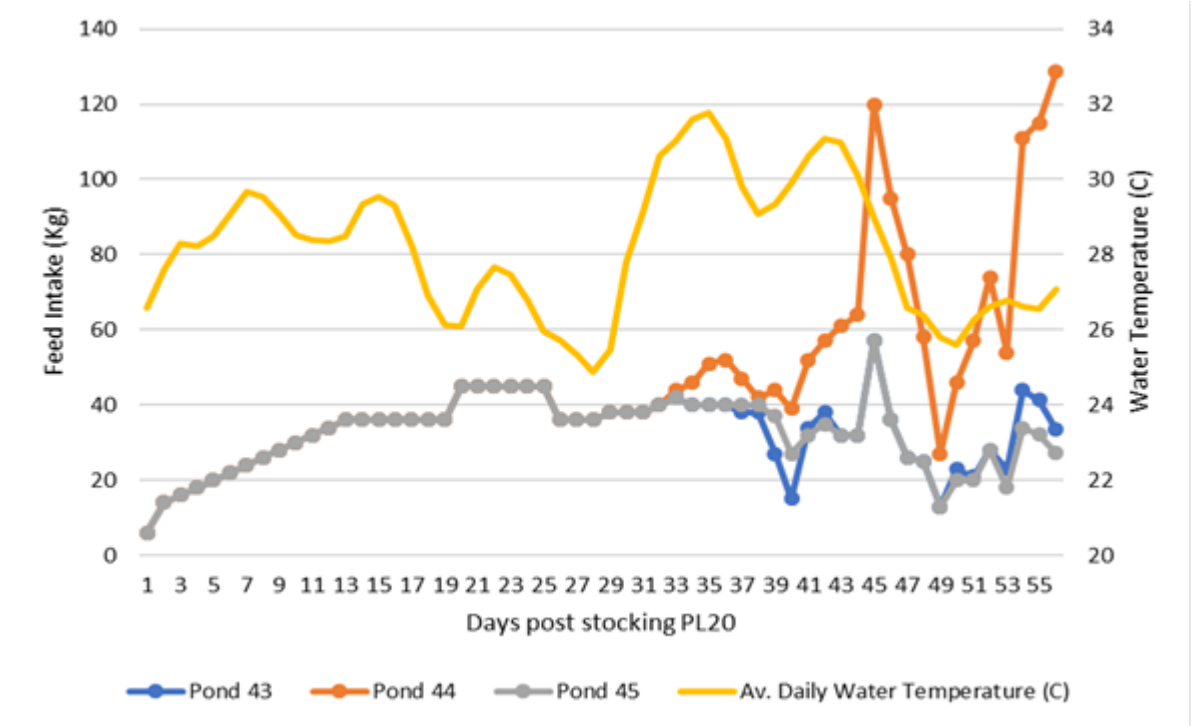

Figure 13. Comparison of daily feed intake (Kg) in trial ponds 43-45 over time following stocking, and average daily water temperature.

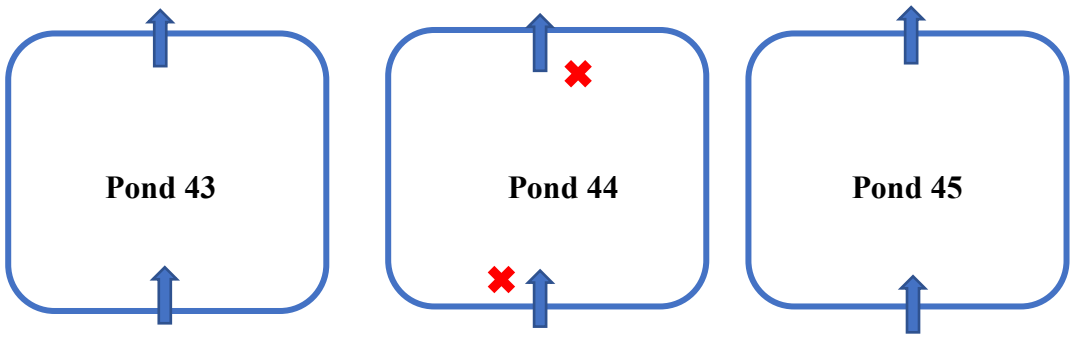

Figure 14. Deployment location of the Empore Disc (ED) passive samplers in amongst the three trial ponds. Red cross indicated the deployment location of the samplers and blue arrows indicate the water inlet and outlet locations in the trial ponds

Figure 15. Position and relative size (1m width x 2m length x 1m depth) of each of the enclosure pens installed into the three trial ponds and stocked with approximately 2000 PLs each.

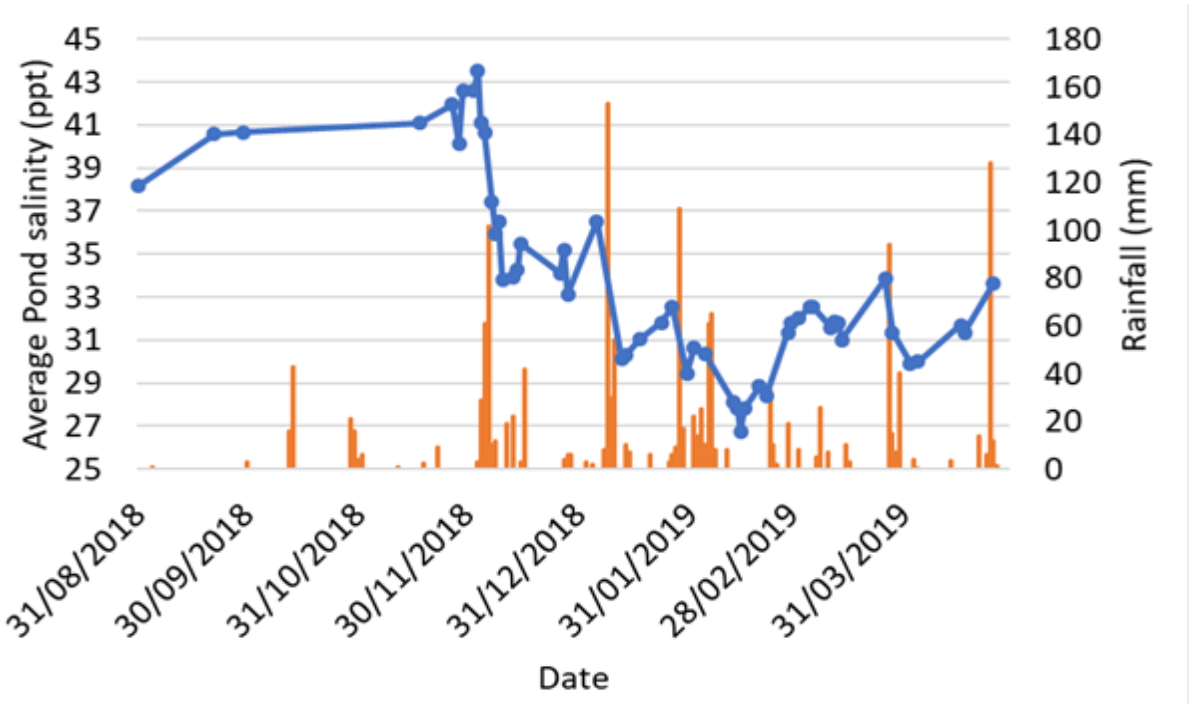

Figure 16. Comparison of the average pond salinity (ppt, blue line) measurements (first 6 weeks post stocking) in commercial grow-out ponds and average daily rainfall (mm, orange bars) recorded at nearby BOM measurement location of Orkabie West Hill (Lat: 21.80° S, Lon: 149.36° E).

Table 16. Flow data from the passive samplers deployed into Pond 44 for the duration of the trial.

CODE	*SITE*	*SITE NAME*	*DATE DEPLOYED*	*DATE RETRIEVED*	*INITIAL MASS (g)*	*FINAL MASS (g)*	*Δ g*	*Normalised Difference*	*DAYS DEPLOYED*	*PFM LOSS RATE (g/day)*	*Average loss (g/day)*	*% error*	***SALINITY (g/L)***	***IONIC STRENGTH (mol/kg)***	***GYPSUM SOLUBILITY (g/L)***	***FLOW VELOCITY (cm/s)***	
1	**1**	**Site 1/ Depl 1**	14/01/19	7/02/19	118.45 121.25	24 38.5	94.45 82.75	13.21	24	3.94 3.45	3.69	 87.61	38	0.8138	0.0198	13.94	0.1394
2	**2**	**Site 2/ Depl 1**	14/01/19	7/02/19	118.85 119.8	48.75 37.4	70.1 82.4	16.13	24	2.92 3.43	3.18	 85.07	38	0.8138	0.0198	11.75	0.1175
3	**1**	**Site 1/ Depl 2**	7/02/19	7/03/19	118.45 117.8	8.4 8.4	110.05 109.4	0.59	28	3.93 3.91	3.92	 100.59	38	0.8138	0.0198	14.90	0.1490
4	**2**	**Site 2/ Depl 2**	7/02/19	7/03/19	119.7 120.8	16.15 24.8	103.55 96	7.57	28	3.70 3.43	3.56	 107.86	38	0.8138	0.0198	13.39	0.1339

Table 17.Analysis of pharmaceuticals and personal care products (PPCPs) chemical compounds that were detected in pond 44 by passive water samplers. N/A = not detected. > 3 BLK = levels are below average blank levels & 3 * Standard deviation of the blank.*

	Blanks			***ng Sampler (Mass) results***								***ng L (water concentration) results***							
Sample Name	**ED Blank 1**	**ED Blank 2**	**Ave+3 *SD Blanks**	**ED Jan 1**	**ED Jan 1***	**ED Jan 2**	**ED Jan 2***	**ED Feb 1**	**ED Feb 1***	**ED Feb 2**	**ED Feb 2***	**ED Jan 1**	**ED Jan 1***	**ED Jan 2**	**ED Jan 2***	**ED Feb 1**	**ED Feb 1***	**ED Feb 2**	**ED Feb 2***
24 D	N/A	0.09	0.09	0.26	0.21	0.35	0.37	3.39	2.90	2.17	2.56	0.09	0.07	0.14	0.14	1.02	0.87	0.69	0.81
Ametryn hydroxy (=Atrz hydroxy)	N/A	N/A		N/A	N/A	1.60	1.10	1.18	1.09	1.15	1.10								
Atrazine	N/A	N/A		0.73	0.55	2.68	1.97	1.63	1.62	1.38	1.12	0.26	0.20	1.05	0.77	0.49	0.49	0.44	0.36
Bromoxynil	N/A	N/A		0.05	0.04	N/A	N/A	N/A	N/A	N/A	N/A								
Desethyl Atrazine	N/A	N/A		0.09	0.12	0.31	0.13	N/A	N/A	0.36	N/A	0.03	0.04	0.12	0.05			0.11	
Desisopropyl Atrazine	N/A	N/A		N/A	N/A	0.18	0.09	N/A	N/A	N/A	N/A								
Diuron	0.05	0.07	0.11	1.15	0.97	3.08	2.35	2.21	2.20	2.31	1.82	0.41	0.35	1.21	0.92	0.66	0.66	0.73	0.58
Hexazinone	N/A	N/A		1.00	0.74	1.91	1.70	2.18	2.07	1.92	1.79	0.36	0.26	0.75	0.66	0.65	0.62	0.61	0.57
MCPA	0.03	0.05	0.09	0.24	0.22	0.27	0.35	1.64	1.34	1.43	1.60	0.08	0.08	0.11	0.14	0.49	0.40	0.45	0.51
Tebuthiuron	N/A	N/A		N/A	0.06	0.11	0.10	N/A	0.05	0.05	0.05		0.02	0.04	0.04		0.02	0.02	0.02

Table 18. Analysis of Neonicitinoid chemical compounds in pond 44 by passive water samplers. N/A = not detected. > 3 BLK = levels are below average blank levels & 3 * Standard deviation of the blank.*

Sample Name	*ED_Blank1*	*ED_Blank2*	*ED_Jan2*	*ED_Jan2**	*ED_Feb1*	*ED_Feb1**	*ED_Feb2*	*ED_Feb2**
Imidacloprid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nitenpyram	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Acetamiprid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Thiacloprid	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Thiamethoxam	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Clothianidin+	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Dinotefuran+	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 19. Analysis of pharmaceuticals and personal care products (PPCPs) chemical compounds that were not detected in pond 44 by passive water samplers. N/A = not detected. > 3 BLK = levels are below average blank levels & 3 * Standard deviation of the blank.*

	Blanks			***ng Sampler (Mass) results***							
Sample Name	**ED Blank 1**	**ED Blank 2**	**Ave+3 *SD Blanks**	**ED Jan 1**	**ED Jan 1***	**ED Jan 2**	**ED Jan 2***	**ED Feb 1**	**ED Feb 1***	**ED Feb 2**	**ED Feb 2***
2,4 DB; 245T; 3,4 DiCl Aniline; Acesulfame D4; Ametryn; Asulam; Atenolol; Atorvastatin; Bromacil; Carbamazepine; Carbofuran; Citalopram; Clopyralid; Codeine; Cotinine; DCPA int std; DCPMU; DCPU 2; Desmethyl Citalopram; Desmethyl Diazepam; Diazinon; Dicamba; Dichlorvos; Diketonitrile; Fenamiphos; Fluazifop; Flumeturon; Fluoxetine; Fluroxypyr; Furosemide; Gabapentin; Haloxyfop +; Haloxyfop -ve; Hydrochlorthiazide; Hydroxycotinine; Ibuprofen; Imazapic; Imazethapyr; Imidacloprid; Iopromide; Malathion; Mecoprop; Metalaxyl; Methiocarb; Methomyl; Metolachlor; Metribuzin; Metsulfuron-Methyl; Naproxen +ve; Nicotine; Pendimethalin; Picloram; Prometryn; Propazine; Propiconazole; Propoxur; Prothioconazole; Pyrimethanil; Sildenafil; Simazine; Simazine hydroxy; Tadalafil; Tebuconazole; Terbuthylazine; Terbuthylazine des ethyl; Terbutryn; Tramadol; Triclopyr; Triclopyr 3; Triclosan; Venlafaxine; Verapamil	N/A	N/A		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

Table 20. Analysis of pharmaceuticals and personal care products (PPCPs) chemical compounds that were detected by passive water samplers but considered likely due to contaminants in deployment, retrieval or sampler handling at the lab. N/A = not detected. > 3 BLK = levels are below average blank levels & 3 * Standard deviation of the blank*

	Blanks			***ng Sampler (Mass) results***							
Sample Name	**ED Blank 1**	**ED Blank 2**	**Ave+3 *SD Blanks**	**ED Jan 1**	**ED Jan 1***	**ED Jan 2**	**ED Jan 2***	**ED Feb 1**	**ED Feb 1***	**ED Feb 2**	**ED Feb 2***
Acesulfame	0.01	0.01	0.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Caffeine	0.91	6.07	14.42	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK
Chlorpyriphos	N/A	0.07		N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
DEET	0.77	0.75	0.80	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK
Paracetamol	0.04	0.09	0.18	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Paraxanthine	0.17	1.23	2.96	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK
Salicylic acid	9.43	7.74	12.17	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK	< 3* BLK

Appendix 2: Epidemiological Data Analysis of Risk Factors Associated with Mortality in the Hatchery Stage of Production.

Table 21. Retrospective epidemiological analysis of the Australian Prawns Farms 2018/19 hatchery farm data set. Data analysis using Microsoft Excel and STATA v15.1. Significance class for data outputs based on the apparent strength of association between a given variable (exposure) and the case (culled tank, 0% survival), reflective of the level of analysis and significance maintained throughout. Scale 10 (highest level of significance) the 0 (lowest level of significance). Unit(s) indicate a value for a given directional effect on mortality. Odds ratio of tank culling (0% survival) from each potential risk factor are shown with significance (p-value).

Category	Potential Risk Factor	Level of Interest	Effect (↑/↔/↓)	Sig Class	Odds Ratio (OR)	Upper (95% CI)	Lower (95% CI)	P value	Summary
Larval Rearing - Husbandry	Merging Tanks	Merged	↑	10	30.03	5.77	156.20	<0.001	The odds of a hatchery tank that was merged to be culled was 30.03 times greater than the odds of a non-merged tank (highly significant, p<0.001). Merging hatchery tanks had a significant detrimental effect on survival.
Larval Rearing - Husbandry	Nauplii Stocking Number	≥900k	↑	10	13.37	2.21	80.90	0.01	The odds of a hatchery tank initially stocked with 900,000 nauplii or more to be culled was 13.37 times greater (highly significant, p<0.02), than the odds of a hatchery tank stocked with less than 900,000 nauplii. Elevated Nauplii Stocking Number had a significant detrimental effect on survival.
Larval rearing - Chemicals/ Therapeutics	Oxytetracycline hydrochloride treatment	At least once	↓	10	0.00	0.00	0.03	<0.001	Treating hatchery tanks with oxytetracycline hydrochloride at least once reduced the odds to be culled by 99.9% (highly significant, p<0.001), compared to not treating. Oxytetracycline treatment had a significant protective effect on survival.
Larval rearing - Chemicals/ Therapeutics	Formalin (Immersion treatment)	At least once	↓	9	0.04	0.00	0.31	>0.05	Treating hatchery tanks with Formalin at least once reduced the odds to be culled by 96.1%, compared to not treating. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity. Formalin treatment had an apparent protective effect on survival.
Larval rearing - Chemicals/ Therapeutics	Formalin (Immersion treatment)	Once	↓	9	0.04	0.00	0.50	>0.05	Treating hatchery tanks with Formalin once reduced the odds to be culled by 96.1%, compared to not treating or treatment more than once. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity. Formalin treatment had an apparent protective effect on survival.
Larval rearing - Chemicals/ Therapeutics	Formalin (Immersion treatment)	Twice	↓	9	0.06	0.00	0.75	>0.05	Treating hatchery tanks with Formalin twice reduced the odds of to be culled by 94.0%, compared to not treating or treatment more than twice. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity. Formalin treatment had an apparent protective effect on survival.
Larval Rearing - Husbandry	Hatchery Run	Fourth	↓	8	0.09	0.02	0.40	>0.05	Hatchery tanks from run 4 had reduced odds to be culled by 94.0%, compared to hatchery tanks from other runs. This association was no longer found to be significant. There was a very strong run effect (temporal effect) that was linked to stocking sequence and therefore time (i.e. no Spatial effect). Hatchery run 4 had an apparent protective effect on survival.
Larval Rearing - Husbandry	Rearing Room	One	↑	8	2.69	1.45	5.01	>0.05	The odds of a hatchery tank from rearing room 1 to be culled was 2.69 times greater than the odds of other rearing rooms. This association was no longer found to be significant. There

									was a very strong run effect (temporal effect) that was linked to stocking sequence and therefore time (i.e. no Spatial effect). Rearing Room 1 had an apparent detrimental effect on survival.
Larval Rearing - Husbandry	Rearing Room	Three	↓	8	0.37	0.20	0.69	>0.05	Hatchery tanks from rearing room 3 had reduced odds to be culled by 62.8%, compared to hatchery tanks from other rearing rooms. This association was no longer found to be significant. There was a very strong run effect (temporal effect) that was linked to stocking sequence and therefore time (i.e. no Spatial effect). Hatchery rearing room 3 had an apparent protective effect on survival.
Larval rearing - Parasites	Ciliated protozoa	Detected	↔	7	-	-	-	>0.05	Hatchery tanks with ciliated protozoal parasites detected during larval rearing were not significantly (p>0.05) associated with an increased probability to be culled.
Larval rearing - Chemicals/ Therapeutics	Erythromycin treatment	Twice or more	↔	7	-	-	-	>0.05	Hatchery tanks that had two or more Erythromycin treatments during larval rearing were not significantly (p>0.05) associated with an increased probability to be culled.
Larval Rearing - Genetics	Northern Territory Broodstock Genetics	Yes	↔	7	-	-	-	>0.05	Hatchery tanks stocked with Northern territory (NT) broodstock genetics were not significantly (p>0.05) associated with an increased probability to be culled.
Larval Rearing - Husbandry	Rearing Room	Two	↔	7	-	-	-	>0.05	Hatchery tanks from rearing room 2 were not significantly (p>0.05) associated with an increased probability to be culled. There was an interaction between hatchery run and rearing room, when accounting for hatchery run the association between rearing room the association was no longer significant.
Larval Rearing - Bacteria/ Toxin	zon occludens toxin gene	Detected	↔	7	-	-	-	>0.05	Hatchery tanks from that detected zon occludens toxin gene during routine pre-stocking sampling were not significantly (p>0.05) associated with an increased probability to be culled.
Larval rearing - Chemicals/ Therapeutics	Erythromycin treatment	Twice or more	↑	6	38.64	11.65	128.16	<0.001	The odds of a hatchery tank treated with erythromycin twice or more times to be culled was 38.64 times greater (significant, p<0.0001), than the odds of a hatchery tank treated erythromycin less times. Multiple erythromycin treatments had a detrimental effect on survival.
Larval rearing - Chemicals/ Therapeutics	Erythromycin treatment	Once	↓	6	0.06	0.01	0.55	0.01	Treating hatchery tanks with erythromycin once reduced the odds to be culled by 94.3% (significant, p<0.02), compared to more or less than one erythromycin treatment. One erythromycin treatment had a protective effect on survival.
Larval rearing - Chemicals/ Therapeutics	Formalin (Immersion treatment)	Once	↓	6	0.01	0.00	0.10	<0.001	Treating hatchery tanks with formalin once reduced the odds to be culled by 94.3% (significant, p<0.001), compared to more or less than one formalin treatment. One formalin treatment had a protective effect on survival.
Larval rearing - Chemicals/ Therapeutics	Formalin (Immersion treatment)	Twice	↓	6	0.02	0.00	0.15	<0.001	Treating hatchery tanks with formalin twice reduced the odds to be culled by 98.0% (significant, p<0.001), compared more or less than two formalin treatments. Multiple formalin treatment had a protective effect on survival.
Larval rearing - Chemicals/ Therapeutics	Formalin (Immersion treatment)	At least once	↓	6	0.01	0.00	0.06	<0.001	Treating hatchery tanks with formalin one or more times reduced the odds to be culled by 98.7% (significant, p<0.001), compared to less than one formalin treatment. Multiple formalin treatment had a protective effect on survival.
Larval Rearing - Husbandry	Hatchery Run	Variable	↑/↓	6	-	-	-	<0.05	Hatchery tanks from runs 1-4 had a significant (p<0.05) association with being culled. No survival observed in hatchery Run 2. No significant difference between Run 1. There was a strong run effect (temporal effect) linked to stocking sequence and therefore time.
Larval Rearing - Husbandry	Hatchery Run	Fourth	↓	6	0.06	0.02	0.21	<0.001	Hatchery tanks from run 4 had reduced the odds to be culled by 94.0% (significant, p<0.001), compared to other runs. Hatchery run 4 had a protective effect on survival.
Larval Rearing - Husbandry	Merging Tanks	Merged	↑	6	8.57	3.25	22.60	<0.001	The odds of a hatchery tank that was merged to be culled was 8.57 times greater than the odds of a non-merged tank (highly significant, p<0.001). Merging hatchery tanks had a

									detrimental effect on survival.
Larval Rearing - Husbandry	Nauplii Stocking Number into Larval rearing tank	≥900k	↑	6	41.32	12.46	137.00	<0.001	The odds of a hatchery tank initially stocked with 900,000 nauplii or more to be culled was 41.32 times greater (significant, p<0.001), than the odds of a hatchery tank stocked with less than 900,000 nauplii. Elevated Nauplii Stocking Number had a detrimental effect on survival.
Larval Rearing - Genetics	Northern Territory Broodstock Genetics	Yes	↑	6	3.39	1.59	7.22	0.001	The odds of a hatchery tank with northern territory genetics to be culled was 3.39 times greater (significant, p<0.002), than the odds of non-northern territory genetic hatchery tanks. Northern Territory genetics had a detrimental effect on survival.
Larval rearing - Chemicals/ Therapeutics	Oxytetracycline hydrochloride treatment	At least once	↓	6	0.01	0.00	0.04	<0.001	Treating hatchery tanks with oxytetracycline one or more times reduced the odds to be culled by 98.9% (significant, p<0.001), compared to less than one oxytetracycline treatment. Oxytetracycline treatments had a protective effect on survival.
Larval rearing - Chemicals/ Therapeutics	Oxytetracycline hydrochloride treatment	Three times or more	↓	6	0.02	0.01	0.06	<0.001	Treating hatchery tanks with oxytetracycline three or more times reduced the odds to be culled by 98.2% (significant, p<0.001), compared to less than three oxytetracycline treatments. Multiple oxytetracycline treatments had a protective effect on survival.
Larval Rearing - Husbandry	Rearing Room	One	↑	6	2.51	1.46	4.32	<0.001	The odds of a hatchery tank from rearing room 1 to be culled was 2.51 times greater (significant, p<0.001), than the odds of other rearing rooms. Rearing Room 1 had a detrimental effect on survival.
Larval Rearing - Husbandry	Rearing Room	Three	↓	6	0.58	0.34	0.97	0.04	Hatchery tanks from rearing room 3 had reduced the odds to be culled by 42.1% (significant, p<0.05), compared to other rearing rooms. Hatchery rearing room 3 had a protective effect on survival.
Larval Rearing - Bacteria/ Toxin	RtX toxin gene	CT Value	↑/↓	6	-	-	-	0.007	Hatchery tank average RtX toxin gene CT value (0-40) was found to significantly (p<0.01) negatively correlate with the number of days between last oxytetracycline treatment and routine sampling. The regression slope displayed a negative linear pattern, different from '0' . r2 - 0.0927. A lower average RtX toxin gene CT value (higher toxin gene 'load') was found to significantly associate with a larger number of days between last oxytetracycline treatment and routine sampling, and vice versa.
Larval Rearing - Bacteria/ Toxin	zon occludens toxin gene	Detected	↓	6	0.21	0.08	0.53	0.001	Hatchery tanks that had zon occludens toxin gene detected during routine sampling qPCR had reduced the odds to be culled by 79.0% (significant, p<0.002), compared to other hatchery tanks that did not have zon occludens toxin gene detected. Detection of zon occludens toxin gene had a protective effect on survival.
Larval Rearing - Husbandry	Hatchery Run	Three	↔	5	-	-	-	>0.05	Hatchery tanks from run 3 were not significantly (p>0.05) associated with an increased probability to be culled. There was a strong run effect (temporal effect) linked to stocking sequence and therefore time.
Larval rearing - Parasites	Ciliated protozoa	Detected	↔	5	-	-	-	>0.05	Hatchery tanks with ciliated protozoal parasites detected during larval rearing were not significantly (p>0.05) associated with an increased probability to be culled.
Larval rearing - Parasites	Ciliated protozoa	Av. score ≥1	↔	5	-	-	-	>0.05	Hatchery tanks with a detected average ciliated protozoal parasite score of 1 or more during larval rearing were not significantly (p>0.05) associated with an increased probability to be culled.
Genetics	East Coast Broodstock Genetics	Yes	↔	5	-	-	-	>0.05	Hatchery tanks stocked with East Coast (EC) broodstock genetics were not significantly (p>0.05) associated with an increased probability to be culled.
Larval Rearing - Bacteria/ Toxin	Pir A toxin gene	Detected	↔	5	-	-	-	>0.05	Hatchery tanks were not found to have any PCR detection of PirA. Samples from 58/260 (23%) hatchery tank populations were tested for PirA toxin via PCR. All 58/58 (100%) PCR negative. Just under half, 23/58 (40%) of the tested hatchery tanks, were culled prior to hatchery harvest.

Larval rearing - Diagnostics	PL Stage at qPCR sampling	Age	↔	5	-	-	-	>0.05	There was no significant association found between each PL age (8-12) at routine sampling and cumulative hatchery survival.
Larval Rearing - Husbandry	Rearing Room	Two	↔	5	-	-	-	>0.05	Hatchery tank from rearing room 2 were not significantly (p>0.05) associated with an increased probability to be culled.
Larval Rearing - Bacteria/ Toxin	RtX toxin gene	Detection	↔	5	-	-	-	>0.05	Hatchery tanks with Rtx toxin gene detected during routine sampling did not significantly (p>0.05) associate with increased probability to be culled. Non-detection of RtX toxin gene in hatchery tanks appeared to perfectly predict being culled. Insufficient data from hatchery tank populations with no RtX toxin gene detected to determine significance.
Larval Rearing - Bacteria/ Toxin	RtX toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average RtX toxin gene CT value (0-40) from routine sampling did not significantly (p>0.05) associate with cumulative hatchery mortality. The regression slope was not different from '0' (p>0.05).
Larval Rearing - Bacteria/ Toxin	RtX toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average RtX toxin gene CT value (0-40) from routine sampling did not significantly (p>0.05) associate with estimated PL population survival at the date of routine sampling. The regression slope was not different from '0' (p>0.05).
Larval Rearing - Bacteria/ Toxin	RtX toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average RtX toxin gene CT value (0-40) from routine sampling did not significantly (p>0.05) associate with the timing of prior erythromycin treatment. The regression slope was not different from '0' (p>0.05).
Larval Rearing - Bacteria/ Toxin	RtX toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average RtX toxin gene CT value (0-40) from routine sampling did not significantly (p>0.05) associate with timing of prior antibiotic treatment. The regression slope was not different from '0' (p>0.05).
Larval rearing - Infrastructure	Tank	Variable	↔	5	-	-	-	>0.05	There was no significant association between individual tank and mortality found.
Larval Rearing - Bacteria/ Toxin	YAFO toxin gene	Detection	↔	5	-	-	-	>0.05	Hatchery tanks with YaFO toxin gene detected during routine sampling did not significantly (p>0.05) associate with increased probability to be culled. Non-detection of YaFO toxin gene in hatchery tanks appeared to perfectly predict being culled. Insufficient data from hatchery tank populations with no YaFO toxin gene detected to determine significance.
Larval Rearing - Bacteria/ Toxin	YAFO toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average YaFO toxin gene CT value (0-40) from routine sampling did not significantly (p>0.05) associate with cumulative hatchery mortality. The regression slope was not different from '0' (p>0.05).
Larval Rearing - Bacteria/ Toxin	YAFO toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average YaFO toxin gene CT value (0-40) did not significantly (p>0.05) associate with estimated PL population survival at date of sampling. The regression slope was not different from '0' (p>0.05).
Larval Rearing - Bacteria/ Toxin	YAFO toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average YaFO toxin gene CT value (0-40) from routine sampling did not significantly (p>0.05) associate with timing of prior oxytetracycline treatment. The regression slope was not different from '0' (p>0.05).
Larval Rearing - Bacteria/ Toxin	YAFO toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average YaFO toxin gene CT value (0-40) from routine sampling did not significantly (p>0.05) associate with timing of prior erythromycin treatment. The regression slope was not different from '0' (p>0.05).
Larval Rearing - Bacteria/ Toxin	YAFO toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average YaFO toxin gene CT value (0-40) from routine sampling did not significantly (p>0.05) associate with timing of prior antibiotic treatment. The regression slope was not different from '0' (p>0.05).
Larval Rearing - Bacteria/ Toxin	zon occludens toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average zon occludens toxin gene CT value (0-40) from routine sampling in PL populations not significantly (p>0.05) associate with cumulative hatchery survival (when above zero). P>0.05, the regression slope was not different from '0'.
Larval Rearing - Bacteria/ Toxin	zon occludens toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average zon occludens toxin gene CT value (0-40) from routine sampling in PL populations did not significantly (p>0.05) associate with estimated PL population

									survival at date of sampling. The regression slope was not different from '0'.
Larval Rearing - Bacteria/ Toxin	zon occludens toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average zon occludens toxin gene CT value (0-40) from routine sampling in PL populationsdid not significantly (p>0.05) associate with the number of days between last oxytetraycline treatment and PL sampling for qPCR analysis. P>0.05, the regression slope was not different from '0'.
Larval Rearing - Bacteria/ Toxin	zon occludens toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average zon occludens toxin gene CT value (0-40) from routine sampling in PL populations did not significantly (p>0.05) associate with the number of days between last erythromycin treatment and PL sampling for qPCR analysis. P>0.05, the regression slope was not different from '0'.
Larval Rearing - Bacteria/ Toxin	zon occludens toxin gene	CT Value	↔	5	-	-	-	>0.05	Hatchery tank average zon occludens toxin gene CT value (0-40) from routine sampling in PL populations did not significantly (p>0.05) associate with the number of days between last antibiotic treatment and PL sampling for qPCR analysis. P>0.05, the regression slope was not different from '0'.
Larval rearing - Chemicals/ Therapeutics	Erythromycin treatment	Number	↑	4	-	-	-	-	Hatchery tanks that were culled were on average more likely to have a lower number of Erythromycin treatments (outside the range of standard error of the mean).
Larval rearing - Chemicals/ Therapeutics	Oxytetracycline hydrochloride treatment	Number	↓	4	-	-	-	-	Hatchery tanks that were culled were on average more likely to have a higher number of Oxytetracycline hydrochloride treatments (outside the range of standard error of the mean).
Larval rearing - Chemicals/ Therapeutics	Formalin (Immersion treatment)	Number	↓	4	-	-	-	-	Hatchery tanks that were culled were on average more likely to have a lower number of Formalin treatments (outside the range of standard error of the mean).
Larval Rearing - Husbandry	Merging Tanks	Yes	↑	4	-	-	-	-	Hatchery tanks that were culled were on average more likely to be merged (outside the range of standard error of the mean).
Larval Rearing - Husbandry	Nauplii Stocking Number into Larval rearing tank	Number	↑	4	-	-	-	-	Hatchery tanks that were culled were on average more likely to be stocked with a higher number of nauplii (outside the range of standard error of the mean).
Larval Rearing - Genetics	Northern Territory Broodstock Genetics	Percentage	↑	4	-	-	-	-	Hatchery tanks that were culled were on average more likely to have a higher percentage of Northern Territory (NT) genetics (outside the range of standard error of the mean).
Genetics	East Coast Broodstock Genetics	Percentage	↓	4	-	-	-	-	Hatchery tanks that were culled were on average more likely to have a lower percentage of East Coast (EC) genetics (outside the range of standard error of the mean).
Larval Rearing - Bacteria/ Toxin	RtX toxin gene	CT value	↑	4	-	-	-	-	Hatchery tanks that were culled were on average more likely to have a higher RtX toxin gene CT value (outside the range of standard error of the mean).
Larval Rearing - Bacteria/ Toxin	YAFO toxin gene	CT value	↑	3	-	-	-	-	Hatchery tanks that were culled were on average more likely to have a higher YaFO toxin gene CT value (outside the range of standard error of the mean).
Larval Rearing - Bacteria/ Toxin	zon occludens toxin gene	CT value	↔	3	-	-	-	-	Hatchery tanks that were culled were not more likely to have a higher toxin gene CT value (within the range of standard error of the mean).
Larval rearing - Parasites	Ciliated protozoa	Score	↔	3	-	-	-	-	Hatchery tanks that were culled were not more likely to have a higher or lower average ciliated protozoa score (within the range of standard error of the mean).
Larval Rearing - Bacteria/ Toxin	Pir A toxin gene	Detection	↔	3	-	-	-	-	Pir A toxin gene was not detected in 23/140 case (>0% survival) populations 35/120 non-case (0% survival) populations.
Larval Rearing - Husbandry	Stocking nauplii into a tank with previous high	Yes	↔	3	-	-	-	-	No significance identified.

	mortality								
Larval rearing - Infrastructure	Tank Location close to rearing room drain location	Yes	↔	3	-	-	-	-	No significance identified.
Larval Rearing - Nutrition	Larval feeding frequency interval	Hours	↔	2	-	-	-	-	Feed every 2–4 h to satiation with high quality feeds (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Nutrition	Artemia concentration	Artemia per mL tank water	↔	2	-	-	-	-	Maintain 3–5 Artemia nauplii/ml in the larval rearing tank so that they are always available to be fed on (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Nutrition	Algae concentration monitoring frequency	Number per day	↔	2	-	-	-	-	At least twice daily (and preferably six times per day), the number of algal cells in each tank should be counted (using a haemocytometer and a microscope) (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Nutrition	Algae concentration	Algae cells per mL tank water	↔	2	-	-	-	-	Maintain from 80-100 cells/mL (Z1-2), 100-130 cells/mL (Z2-3), 80-100 cells/mL (Mysis), 60-80 cells/mL (Pl1-3) (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Nutrition	Algae feeding frequency interval	Number per day	↔	2	-	-	-	-	Perform algae addition to larval rearing tanks multiple times per day (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Water Quality	Unionised ammonia concentration	mg/L	↔	2	-	-	-	-	Unionised ammonia (NH_3) concentration should be maintained at less than 0.1 mg/L (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Water Quality	salinity	ppt	↔	2	-	-	-	-	Maintain salinity levels of 30-35 ppt for *Penaeus monodon* larvae up until PL10-12, when they can begin acclimation to pond salinity conditions (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Water Quality	pH	units	↔	2	-	-	-	-	pH levels should be maintained between 7.8-8.2 (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Husbandry	Stocking density	Nauplii per L	↔	2	-	-	-	-	Stocking density should be between 75 and 120 nauplii per litre, assuming a full larval–rearing tank. (FAO Fisheries and Aquaculture Department, 2007).
Larval rearing - Parasites	Ciliated protozoa	Number	↔	1	-	-	-	-	Ciliated protozoa identified in multiple tanks but not consistently present in tanks experiencing mortality and absent in tanks not experiencing mortality. Histopathology did not detect any associated pathology, nor the parasite in sections. Unknown significance.
Larval Rearing - Husbandry	Routine health monitoring	Frequency	↔	1	-	-	-	-	Routine full health assessment of larvae using the microscope should performed to monitor feed intake, growth / performance, identify early signs of disease and facilitate sampling.
Larval Rearing - Water Quality	Daily water exchange	Percent	↔	1	-	-	-	-	Daily water exchange was not commenced until post larval stages. Sub-optimal water quality conditions were detected prior to water exchange commencing, suggesting need for earlier commencement.
Larval Rearing - Husbandry	Bacterial plate counts	CFU per mL	↔	1	-	-	-	-	Routine bacterial monitoring of algae and water inputs did not appear to align with mortality. Routine bacterial monitoring other feed inputs not performed.
Larval rearing - Infrastructure	Black plastic hatchery tank covering	PL age	↔	1	-	-	-	-	Timing of removal of black plastic hatchery tank coverings did not appear to align with mortality. Insufficient data available to allow analysis.
Broodstock - Viruses	Decapod Hepandensovirus 1 (HDV)	Detection	↔	1	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 44% (4/9) Not detected - 56% (5/9). Comparison group. No significance identified.

Larval Rearing - Nutrition	Feeding Frozen and thawed Artemia	Yes	↔	1	-	-	-	-	Maintaining frozen thawed artemia could be a potential source of bacterial contamination and have a negative impact on nutrient quality. Changes in protocol during the hatchery season and insufficient data collection limits ability to perform data analysis.
Broodstock - Viruses	Gill associated virus (GAV)	Detection	↔	1	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 100% (9/9); Not detected - 0% (0/9). No comparison groups. Present in all spawning batches. No significance identified.
Larval Rearing - Viruses	Gill-associated virus (GAV)	Detection	↔	1	-	-	-	-	Submission Reference: AAHL: 19-00191. Tacman PCR (de la Vega). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). A greater proportion of case (0% survival) PL population did not have GAV detected. Detected - 71% (7/9) Not detected - 29% (2/9). Test results not surprising given all broodstock batches positive. No comparison group. No significance identified.
Broodstock - Viruses	Infectious hypodermal and hematopoietic necrosis (IHHNV)	Detection	↔	1	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 100% (9/9); Not detected - 0% (0/9). No comparison groups. Present in all spawning batches. No significance identified.
Broodstock - Viruses	Wenzhou shrimp virus-2 (When 2)	Detection	↔	1	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 71% (7/9) Not detected - 29% (2/9). Comparison group. No significance of this virus found in peer reviewed literature. No significance identified.
Larval Rearing - Viruses	Gill-associated virus (GAV)	Detection	↔	1	-	-	-	-	Submission Reference: P18-04857. Collection Date: 27/09/18. Hatchery populations experiencing mortality (Run. Tank ID): 3.3, 2.21, 2.45, 2.49. GAV detected in 25% (1/4) of samples. No comparison group. No significance identified.
Larval Rearing - Viruses	Infectious hypodermal and hematopoietic necrosis (IHHNV)	Detection	↔	1	-	-	-	-	Submission Reference: P18-04857. Collection Date: 27/09/18. Hatchery populations experiencing mortality (Run.Tank ID): 3.3, 2.21, 2.45, 2.49. IHHNV detected in 50% (2/4) of samples. No comparison group. No significance identified.
Larval Rearing - Bacteria	*Vibrio* sp., *Bacillus* sp., *Micrococcus* sp.	Detection	↔	1	-	-	-	-	Submission Reference: P18-04904. Collection Date: 26/09/18. Bacterial culture of sterile saline flushed tank scraping. Mixed growth bacteria. No significance identified.
Larval Rearing - Water Quality	Water temperature in hatchery tanks	Celsius	↔	0	-	-	-	-	Water temperature in the hatchery of 28–30°C, can be up to 32°C for zoea (FAO Fisheries and Aquaculture Department, 2007).
Larval Rearing - Additives	Probiotics	Yes	↔	0	-	-	-	-	Insufficient Data to include in Analysis
Larval Rearing - Water Quality	Acclimatisation of nauplii	Yes	↔	0	-	-	-	-	Insufficient Data to include in Analysis
Broodstock - Bacteria/Toxin	Acute Hepatopancreatic Necrosis Disease (AHPND) Pir A	Detection	↔	0	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 0% (0/9); Not detected - 100% (9/9). No comparison group. No significance identified.
Larval rearing - Bacteria/Toxin	AHPND VpPirA	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. TaqMan PCR (Han et al). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Larval Rearing - Viruses	Covert mortality nodavirus	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. Tacman PCR (Pooljun et al., 2016). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Larval Rearing - Microsporidian	Enterocytozoon hepatopenaei	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. nested PCR. Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6);

									Not detected - 100% (6/6). No comparison group. No significance identified.
Broodstock - Microsporidian	Enterocytozoon hepatopenaei (EHP)	Detection	↔	0	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 0% (0/9); Not detected - 100% (9/9). No comparison group. No significance identified.
Larval Rearing - Viruses	Infectious Myonecrosis Virus	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. Tacman PCR (OIE). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Larval Rearing - Viruses	Laem-Singh Virus	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. RT nested PCR. Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Broodstock - Viruses	Mourilyan virus (MoV)	Detection	↔	0	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 0% (0/9); Not detected - 100% (9/9). No comparison group. No significance identified.
Larval Rearing - Bacteria	Necrotising hepatopancreatitis bacterium	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. Tacman PCR (OIE). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Larval Rearing - Viruses	Shrimp hemocyte iridescent virus	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. TaqMan PCR (Qui et al. 2018). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Larval Rearing - Viruses	Taura Syndrome Virus (TSV)	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. Tacman PCR (OIE). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Broodstock - Viruses	Taura Syndrome Virus (TSV)	Detection	↔	0	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 0% (0/9); Not detected - 100% (9/9). No comparison group. No significance identified.
Larval Rearing - Viruses	White Spot Syndrome Virus	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. TaqMan PCR (CSIRO). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Broodstock - Viruses	White Spot Syndrome Virus (WSSV)	Detection	↔	0	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 0% (0/9); Not detected - 100% (9/9). No comparison group. No significance identified.
Larval Rearing - Viruses	Yellow head virus (Genotype 1)	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. Taqman PCR (AFDL). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Larval Rearing - Viruses	Yellow head virus (Genotype 7)	Detection	↔	0	-	-	-	-	Submission Reference: AAHL: 19-00191. Taqman PCR (AFDL). Convenience sampling of 6 PL populations from Run 3 experiencing mortality (26, 27, 30, 36, 37, 38). Detected - 0% (0/6); Not detected - 100% (6/6). No comparison group. No significance identified.
Broodstock - Viruses	Yellowhead virus 7 (YHV7)	Detection	↔	0	-	-	-	-	Broodstock pleopod PCR testing (Pooled results each spawning batch). Detected - 0% (0/9); Not detected - 100% (9/9). No comparison group. No significance identified.
Larval Rearing - Viruses	Yellow head virus (Genotype 7)	Detection	↔	0	-	-	-	-	Submission Reference: P18-04857. Collection Date: 27/09/18. Hatchery populations (Run.Tank ID): 3.3, 2.21, 2.45, 2.49. Not detected - 100% (4/4). No comparison group. No significance identified.
Larval Rearing - Viruses	Mourilyan virus (MoV)	Detection	↔	0	-	-	-	-	Submission Reference: P18-04857. Collection Date: 27/09/18. Hatchery populations (Run.Tank ID): 3.3, 2.21, 2.45, 2.49. Not detected - 100% (4/4). No comparison group. No significance identified.

Appendix 3: Epidemiological Data Analysis of Risk Factors Associated with Mortality in the Grow-out Stage of Production.

Table 22. Retrospective epidemiological analysis of the Australian Prawns Farms 2018/19 grow-out farm data set. Data analysis using Microsoft Excel and STATA v15.1. Significance class for data outputs based on the apparent strength of association between a given variable (exposure) and the case (culled pond, 0% survival), reflective of the level of analysis and significance maintained throughout. Scale 10 (highest level of significance) the 0 (lowest level of significance). Unit(s) indicate a value for a given directional effect on mortality. Odds ratio of pond culling (0% survival) from each potential risk factor are shown with significance (p-value).

Category	Potential Risk Factor	Level of Interest	Effect (↑/↔/↓)	Sig Class	Odds Ratio (OR)	Upper (95% CI)	Lower (95% CI)	P value	Summary
Post Stocking - Water Quality	Nitzschia sp. during First 30 days Post Stocking	Max score ≥ 5	↑	10	61.01	4.90	759.58	<0.001	The odds of a grow-out pond that had a maximum Nitzschia score of 5 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 61.0 times greater (highly significant, p<0.001), than the odds of a grow-out pond that had a maximum nitzschia score below 5. Detection of elevated levels of nitzschia had a significant detrimental effect on survival.
Pond Preparation - Husbandry	Days between completion of initial pond filling and PL stocking	11 days or more	↓	10	0.01	0.00	0.09	<0.001	Stocking grow-out ponds with PLs 11 days or more after completion of pond filling reduced the odds to be culled by 99.9% (highly significant, p<0.001), compared to stocking PLs into grow-out ponds less than 11 days after completion of pond filling. Increasing the time between pond filling and PL stocking had a significant protective effect on survival.
Post Stocking - Water Quality	Salinity during the First 6 weeks Post Stocking	Min ≥36 ppt	↓	10	0.01	0.00	0.09	<0.001	Grow-out ponds with a minimum salinity of 36ppt or higher during the first 6 weeks post stocking had reduced odds to be culled by 98.6% (highly significant, p<0.001), compared grow-out ponds with a minimum salinity below 36ppt. Maintaining elevated grow-out pond salinity in the post stocking period had a significant protective effect on survival.
Post Stocking - Water Quality	Cyanobacteria sp. during the First 30 days Post Stocking	Av score ≥ 8	↓/↔	9	0.08	0.01	0.78	>0.05	Grow-out ponds with an average cyanobacteria score of 8 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking had reduced odds to be culled by 98.6%, compared grow-out ponds with a minimum salinity below 36ppt. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity. Elevated grow-out pond salinity in the post stocking period had an apparent protective effect on survival.
Post Stocking - Water Quality	Gymnodinium sp. during the First 30 days Post Stocking	Max score ≥ 5	↑/↔	9	31.88	3.47	292.56	>0.05	The odds of a grow-out pond that had a maximum Gymnodinium score of 5 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 31.88 times greater, than the odds of a grow-out pond that had a maximum Gymnodinium score below 5. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity.

									Detection of elevated levels of Gymnodinium had an apparent detrimental effect on survival.
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Av Daily Change ≥ 0.15 units	↑/↔	9	60.67	6.63	555.49	>0.05	The odds of a grow-out pond that had an average daily pH change of 0.15 units or higher during the post stocking period (30 days), to be culled, was 60.67 times greater, than the odds of a grow-out pond with an average daily pH change of less than 0.15. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity. Increased average daily pH change had an apparent detrimental effect on survival.
Stocking - Husbandry	PL Age at Stocking	Min age PL10 or older	↓/↔	9	0.13	0.02	0.92	>0.05	Stocking grow-out ponds with PL10 or older reduced the odds to be culled by 86.7%, compared to stocking PLs less than PL10. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity. Increasing the age PLs being stocked had a significant protective effect on survival.
Post Stocking - Water Quality	Salinity during the First 6 weeks Post Stocking	Max change ≥ 6 ppt	↑/↔	9	29.54	2.07	422.16	>0.05	The odds of a grow-out pond that had a maximum salinity change of 6 ppt or higher during the post stocking period (6 weeks), to be culled, was 29.54 times greater, than the odds of a grow-out pond that had a maximum salinity change less than 6 ppt. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity. Increased maximum salinity change during the post stocking period had an apparent detrimental effect on survival.
Hatchery - Bacteria/ Toxin	zon occludens toxin gene CT value	Av CT ≥ 34	↓/↔	9	30.10	2.56	353.89	>0.05	The odds of a grow-out pond that had an average zon occludens toxin gene CT value of 34 or higher from routine hatchery sampling, to be culled, was 30.10 times greater than the odds of a grow-out pond that had an average zon occludens toxin gene CT value of less than 34. This association was no longer found to be significant when assessing confounding variable interactions, which identified collinearity. Higher average zon occludens toxin gene CT value (i.e. lower toxin gene quantity) in hatchery PLs stocked into grow-out ponds had an apparent detrimental effect on survival.
Post Stocking - Water Quality	Gymnodinium sp during the First 30 days Post Stocking	Av score ≥ 1	↑/↔	8	17.47	2.67	114.34	>0.05	The odds of a grow-out pond that had an average Gymnodinium score of 1 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 17.47 times greater, than the odds of a grow-out pond that had an average Gymnodinium score below 1. This association was no longer found to be significant when assessing confounding variable interactions and pearson's correlation (>0.9). Detection of elevated levels of Gymnodinium had an apparent detrimental effect on survival.
Post Stocking - Water Quality	Nitzschia sp. during the First 30 days Post Stocking	Av score ≥ 2	↑/↔	8	12.64	1.89	84.45	>0.05	The odds of a grow-out pond that had an average Nitzschia score of 2 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 12.64 times greater, than the odds of a grow-out pond that had an average Nitzschia score below 2. This association was no longer found to be significant when assessing confounding variable interactions and pearson's correlation (>0.9). Detection of elevated levels of Nitzschia had an apparent detrimental effect on survival.
Pond Preparation - Husbandry	Days Between initial Pond Fertilisation and Stocking	10 days or more	↓/↔	8	0.05	0.00	0.62	>0.05	Stocking grow-out ponds with PLs 10 days or more after fertilisation reduced the odds to be culled by 99.4%, compared to stocking PLs into grow-out ponds less than 10 days after fertilisation. This association was no longer found to be significant when assessing confounding variable interactions and pearson's correlation (>0.65). Increasing the time between fertilisation and PL stocking had an apparent protective effect on survival.
Acclimation - Water Quality	Dissolved Oxygen during Acclimation	Max ≥ 10 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds that had an acclimation (3 hours) maximum DO of 10 mg/L or higher did not significantly (p>0.05) associate with being culled.

	(3 hours)								
Hatchery - Chemicals/ Therapeutics	Oxytetracycline (Immersion) treatment	One or more	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PLs treated with Oxytetracycline 1 or more times in the hatchery did not significantly associate with being culled. All (8/8) Grow-out populations from hatchery tanks with no Oxytetracycline exposure in the hatchery predicted survival 100%
Hatchery - Chemicals/ Therapeutics	Oxytetracycline (Immersion) treatment	Four or more	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PLs treated with Oxytetracycline 4 or more times in the hatchery did not significantly associate with being culled.
Acclimation - Water Quality	pH during Acclimation (3 hours)	Max ≥ 8.7 pH	↔	7	-	-	-	>0.05	Grow-out ponds that had an acclimation (3 hours) maximum pH of 8.7 or higher did not significantly (p>0.05) associate with being culled.
Acclimation - Water Quality	Water Temperature during Acclimation (3 hours)	Max ≥ 27°C	↔	7	-	-	-	>0.05	Grow-out ponds that had an acclimation (3 hours) maximum temperature of 27°C or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Max daily change ≥ 3°C	↔	7	-	-	-	>0.05	Grow-out ponds that over the post stocking period (30 days) had a maximum daily water temperature of 3°C or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Max ≥ 31°C	↔	7	-	-	-	>0.05	Grow-out ponds that during the post stocking period (30 days) had a maximum water temperature of 31°C or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Min ≥ 24°C	↔	7	-	-	-	>0.05	Grow-out ponds that over the post stocking period (30 days) had a minimum daily temperature of 24°C or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Total Ammonia Nitrogen (TAN) during the First 30 days Post Stocking	Av ≥ 0.5 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds that had an average TAN of 0.5 mg/L or higher over the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Cyanobacteria sp. during the First 30 days Post Stocking	Max score ≥ 10	↔	7	-	-	-	>0.05	Grow-out ponds that during the post stocking period (30 days) had a maximum cyanobacteria score of 10 (0=absent; 5=low; 10=medium; 15=high) or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Max daily change ≥ 9 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds that during the post stocking period (30 days) had a maximum daily DO change of 9 mg/L or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Max ≥ 13 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds that during the post stocking period (30 days) had a maximum daily DO of 13 mg/L or higher did not significantly (p>0.05) associate with being culled.
Acclimation - Water Quality	Dissolved Oxygen (DO) during acclimation (3 hours) (Minimum)	≥ 8 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds that had a minimum acclimation DO of 8 mg/L or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post	Av daily change ≥ 5 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds with an average daily DO change of 5 mg/L or higher during the post stocking period (30 days) did not significantly associate (p>0.05) with being culled.

	Stocking								
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Av daily ≥ 7.5 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds with an average daily DO of 7.5 mg/L or higher during the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Min ≥ 4 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds that over the post stocking period (30 days) had a minimum daily DO of 4.0 mg/L or higher did not significantly (p>0.05) associate with being culled.
Hatchery - Chemicals/ Therapeutics	Erythromycin (Immersion) treatment	One or more	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PLs treated with Erythromycin in the hatchery did not significantly (p>0.05) associate with being culled. There were no grow-out populations without exposure to Erythromycin during larval rearing, which limited the comparative analysis.
Hatchery - Chemicals/ Therapeutics	Erythromycin (Immersion) treatment	Two or more	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PLs treated with Erythromycin in the hatchery did not significantly (p>0.05) associate with being culled. This may be explained by the temporal influence on mortality.
Hatchery - Performance	Hatchery (Larval Rearing) Survival	Av Percent	↔	7	-	-	-	>0.05	Grow-out ponds stocked in PLs from hatchery tanks that had an average (larval rearing) survival of greater than or equal to 10% (or 20% or 30% or 40%) were not significantly (p>0.05) associated with being culled. Note that only hatchery (larval rearing) tanks that survived could be analysed (Survival bias).
Hatchery - Performance	Hatchery (Larval Rearing) Survival	Max Percent	↔	7	-	-	-	>0.05	Grow-out ponds stocked in PLs from hatchery tanks that had a maximum (larval rearing) survival of greater than or equal to 30% (or 40% or 50% or 60%) were not significantly (p>0.05) associated with being culled. Note that only hatchery (larval rearing) tanks that survived could be analysed (Survival bias).
Hatchery - Performance	Hatchery (Larval Rearing) Survival	Min Percent	↔	7	-	-	-	>0.05	Grow-out ponds stocked in PLs from hatchery tanks that had a minimum (larval rearing) survival of greater than or equal to 10% (or 20% or 30% or 40%) did not significantly (p>0.05) associate with being culled. Note that only hatchery (larval rearing) tanks that survived could be analysed (Survival bias).
Stocking - Husbandry	Hatchery Run (Number)	Three	↔	7	-	-	-	>0.05	Grow-out ponds that were stocked with PLs from hatchery run 3 did not significantly (p>0.05) associate with being culled. The original association may be explained by the temporal influence on mortality.
Stocking - Husbandry	Individual Hatchery Tanks used for pond stocking	Five or more	↔	7	-	-	-	>0.05	Grow-out ponds that were stocked with 5 or more hatchery tanks did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Mixed Dinoflagellates during the First 30 days Post Stocking	Max score ≥ 5	↔	7	-	-	-	>0.05	Grow-out ponds that had a maximum mixed dinoflagellates score of 5 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Mixed Dinoflagellates sp. during the First 30 days Post Stocking	Av score ≥ 4	↔	7	-	-	-	>0.05	Grow-out ponds that had an average mixed dinoflagellate score of 4 or higher (0=absent; 5=low; 10=medium; 15=high) or higher over the first 30 days post stocking did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Av daily ≥ 8.3 units	↔	7	-	-	-	>0.05	Grow-out ponds with an average daily pH of 8.3 or higher over the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	pH during the First 30 days Post	Max daily	↔	7	-	-	-	>0.05	Grow-out ponds that during the post stocking period (30 days) had a maximum daily pH change of 0.4 or higher did not significantly (p>0.05) associate with being culled.

	Stocking	change ≥ 0.4 units							
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Max ≥ 8.9 units	↔	7	-	-	-	>0.05	Grow-out ponds that during the post stocking period (30 days) had a maximum daily pH of 8.9 or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Min ≥ 8 units	↔	7	-	-	-	>0.05	Grow-out ponds that over the post stocking period (30 days) had a minimum daily pH of 8.0 or higher did not significantly (p>0.05) associate with being culled.
Stocking - Husbandry	PL Age at Stocking	Av age PL11 or older	↔	7	-	-	-	>0.05	The average age of PLs stocked into Grow-out ponds did not significantly (0<0.05) associate with being culled. This was also assessed at PL 8; 9;10;11;12 with no significance determined.
Stocking - Husbandry	PL Age at Stocking	Max age PL13 or older	↔	7	-	-	-	>0.05	Grow-out ponds stocked in PL 13 or older did not significantly (p>0.05) associate with being culled.
Infrstructure - Ponds	Pond Size/Age	Less than 12 ML	↔	7	-	-	-	>0.05	Grow-out ponds that are smaller (<12ML)/ newer did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	RtX toxin gene CT value	Av Ct ≥ 32	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PL that had an average RtX toxin gene CT value of 32 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	RtX toxin gene CT value	Min CT ≥ 30	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PL that had a minimum RtX toxin gene CT value of 30 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Salinity during the First 6 weeks Post Stocking	Max ≥ 41 ppt	↔	7	-	-	-	>0.05	Grow-out ponds that over the post stocking period (6 weeks) had a maximum salinity of 41 ppt or higher did not significantly (p>0.05) associate with being culled.
Stocking - Husbandry	Stocking Density	≥ 70 PL per sqm	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PL at a density of 70 per sqm or higher did not significantly (p>0.05) associate with being culled.
Pond Preparation - Husbandry	Stocking PLs into grow-out ponds over an extended period	9 days or more	↔	7	-	-	-	>0.05	Grow-out ponds stocked PLs over an extended period of 9 or more days did not significantly (p>0.05) associate with being culled. There was a strong temporal influence of data, with 100% (20/20) of Run 1 and 3 stockings were performed over less than 9 days.
Post Stocking - Water Quality	Total Ammonia Nitrogen (TAN) during the First 30 days Post Stocking	Max ≥ 1.5 mg/L	↔	7	-	-	-	>0.05	Grow-out ponds that over the post stocking period (30 days) had a maximum total ammonia nitrogen of 1.5 mg/L or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Av daily ≥ 27.5°C	↔	7	-	-	-	>0.05	Grow-out ponds with an average temperature of 27.5°C or higher over the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Av daily change ≥ 2°C	↔	7	-	-	-	>0.05	Grow-out ponds with a daily temperature change of 2°C or higher over the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	YAFO toxin gene CT value	Av CT ≥ 32	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PL that had an average YaFO toxin gene CT value of 32 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	YAFO toxin gene CT value	Min CT ≥ 30	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PL that had a minimum YaFO toxin gene CT value of 30 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.

Hatchery - Bacteria/ Toxin	zon occludens toxin gene CT value	Min CT ≥ 34	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PL that had a minimum zon occludens toxin gene CT value of 34 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	zon occludens toxin gene	Detected	↔	7	-	-	-	>0.05	Grow-out ponds stocked with PL that had a zon occludens toxin gene detected, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Chemicals/ Therapeutics	Oxytetracycline (Immersion) treatment	Four or more	↑	6	15.20	1.77	130.41	0.013	The odds of a grow-out pond stocked with PLs treated with oxytetracycline four or more times in the hatchery, to be culled, was 15.2 times greater (significant, p<0.02) than the odds of a grow-out pond stocked with PL treated with oxytetracycline less times. Multiple hatchery oxytetracycline treatments had an apparent detrimental effect on grow-out survival.
Pond Preparation - Husbandry	Days Between initial Pond Fertilisation and Stocking	10 days or more	↓	6	0.03	0.01	0.16	<0.001	Stocking grow-out ponds with PLs 10 days or more after fertilisation reduced the odds to be culled by 96.9% (significant, p<0.001), compared to stocking PLs into grow-out ponds less than 10 days after fertilisation. Increasing the time between pond fertilisation and PL stocking had an apparent protective effect on survival.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Max daily change ≥ 9 mg/L	↑	6	6.19	1.19	32.23	0.03	The odds of a grow-out pond that had a maximum daily DO change of 9mg/L or higher during the post stocking period (30 days), to be culled, was 6.19 times greater (significant, p<0.05) than the odds of a grow-out pond that had a maximum daily DO change less than 9mg/L. Increased maximum daily DO change in the post stocking period had an apparent detrimental effect on grow-out survival.
Hatchery - Chemicals/ Therapeutics	Erythromycin (Immersion) treatment	Two or more	↓	6	0.04	0.01	0.24	<0.001	Stocking grow-out ponds with PLs treated with Erythromycin two or more times in the hatchery, reduced the odds to be culled by 95.7% (significant, p<0.001), compared to grow-out ponds stocked with PLs treated less than two times in the hatchery. Multiple hatchery erythromycin treatments had an apparent protective effect on grow-out survival.
Post Stocking - Water Quality	Gymnodinium sp during the First 30 days Post Stocking	Av score ≥ 1	↑	6	32.00	6.30	162.53	<0.001	The odds of a grow-out pond that had an average Gymnodinium score of 1 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 32.00 times greater (significant, p<0.001) than the odds of a grow-out pond that had an average Gymnodinium score of less than 1. Increased Gymnodinium in post stocking period had an apparent detrimental effect on grow-out survival.
Post Stocking - Water Quality	Gymnodinium sp. during the First 30 days Post Stocking	Max score ≥ 5	↑	6	45.33	8.14	252.33	<0.001	The odds of a grow-out pond that had a maximum Gymnodinium score of 5 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 45.33 times greater (significant, p<0.001) than the odds of a grow-out pond that had an average Gymnodinium score of less than 5. Increased Gymnodinium in post stocking period had an apparent detrimental effect on grow-out survival.
Stocking - Husbandry	Hatchery Run (Number)	Three	↓	6	0.19	0.04	0.84	0.028	Stocking grow-out ponds with PLs from hatchery run 3, reduced the odds to be culled by 81.25% (significant, p<0.05), compared to stocking PLs into grow-out ponds not from run 3. Stocking grow-out ponds with PLs from hatchery run 3 had an apparent protective effect on survival.
Post Stocking - Water Quality	Nitzschia sp. during the First 30 days Post Stocking	Av score ≥ 2	↑	6	24.00	4.99	115.36	<0.001	The odds of a grow-out pond that had an average Nitzschia score of 2 or more (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 24.00 times greater (significant, p<0.001) than the odds of a grow-out pond that had an average Nitzschia score of less than 5. Increased Nitzschia in post stocking period had an apparent detrimental effect on grow-out survival.
Post Stocking - Water Quality	Nitzschia sp during the First 30 days Post Stocking (Maximum score)	Max score ≥ 5	↑	6	78.00	8.31	732.03	<0.001	The odds of a grow-out pond that had a maximum Nitzschia score of 5 or more (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking, to be culled, was 78.00 times greater (significant, p<0.001) than the odds of a grow-out pond that had a maximum Nitzschia score of less than 5. Increased Nitzschia in post stocking

									period had an apparent detrimental effect on grow-out survival.
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Av daily change ≥ 0.15 units	↑	6	60.67	6.63	555.49	p<0.001	The odds of a grow-out pond that had an average daily change in pH by 0.15 units or more during the post stocking period (30 days), to be culled, was 60.67 times greater (significant, p<0.001) than the odds of a grow-out pond that had an average daily change in pH by less than 0.15 units. Increased average daily pH change in the post stocking period had an apparent detrimental effect on grow-out survival.
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Max daily change ≥ 0.4 units	↑	6	9.78	2.21	43.33	0.003	The odds of a grow-out pond that had a maximum daily change in pH by 0.4 units or more during the post stocking period (30 days), to be culled, was 9.78 times greater (significant, p<0.005) than the odds of a grow-out pond that had a maximum daily change in pH by less than 0.4 units. Increased average daily pH change in the post stocking period had an apparent detrimental effect on grow-out survival
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Max ≥ 8.9 units	↑	6	10.23	1.89	55.33	0.007	The odds of a grow-out pond that had a maximum pH of 8.9 units or more during the post stocking period (30 days), to be culled, was 10.23 times greater (significant, p<0.01) than the odds of a grow-out pond that had a maximum pH less than 8.9 units. Increased maximum pH change in the post stocking period had an apparent detrimental effect on grow-out survival.
Infrastructure - Ponds	Pond Size	Less than 12 ML	↑	6	9.69	1.87	50.19	0.007	The odds of a grow-out pond of a volume less than or equal to12 ML, to be culled, was 9.69 times greater (significant, p<0.01) than the odds of a grow-out pond of a volume greater than 12 ML. Smaller volume grow-out ponds had an apparent detrimental effect on grow-out survival.
Pond Preparation - Husbandry	Stocking PLs into grow-out ponds over an extended duration	9 days or more	↑	6	6.19	1.19	32.23	0.03	The odds of a grow-out pond stocked with PLs over 9 or more days, to be culled, was 6.19 times greater (significant, p<0.05) than the odds of a grow-out pond stocked over less than 9 days. Increasing the number of days, a grow-out pond was stocked over had an apparent detrimental effect on grow-out survival.
Post Stocking - Water Quality	Salinity over the First 6 weeks Post Stocking	Max change ≥ 6 ppt	↑	6	48.29	5.36	435.13	0.001	The odds of a grow-out pond that had a maximum salinity change of 6 ppt or more during the post stocking period (6 weeks), to be culled, was 48.29 times greater (significant, p<0.002) than the odds of a grow-out pond that had a maximum salinity change less than 6 ppt. Increased salinity change in the post stocking period had an apparent detrimental effect on grow-out survival
Pond Preparation - Husbandry	Days Between completion of initial pond filling and Stocking	11 days or more	↓	6	0.01	0.00	0.09	<0.001	Stocking grow-out ponds with PLs 11 or more days after pond filling, reduced the odds to be culled by 99.0% (significant, p<0.001), compared to stocking PLs into grow-out ponds less than 11 days after pond filling. Stocking grow-out ponds with PLs following increased from time of pond filling had an apparent protective effect on survival.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Min ≥ 4 mg/L	↓	6	0.22	0.05	0.93	0.04	Grow-out ponds with a minimum dissolved oxygen levels of 4mg/L or more during the first 30 days post stocking, had reduced the odds to be culled by 77.9% (significant, p<0.05), compared to grow-out ponds with a minimum dissolved oxygen levels below 4mg/L. Increased post stocking minimum dissolved oxygen had an apparent protective effect on survival.
Post Stocking - Water Quality	Mixed Dinoflagellates sp. during the First 30 days Post Stocking	Av score ≥ 4	↓	6	0.10	0.02	0.53	0.007	Grow-out ponds with an average mixed dinoflagellate score of 4 or more (0=absent; 5=low; 10=medium; 15=high) during the first 30 days post stocking, had reduced the odds to be culled by 89.7% (significant, p<0.01), compared to grow-out ponds with an average mixed dinoflagellate score below 4. Increased levels of mixed dinoflagellate post stocking had an apparent protective effect on survival.
Stocking - Husbandry	PL Age at Stocking	Max age PL13 or	↓	6	0.06	0.01	0.51	0.011	Stocking grow-out ponds with PLs of a maximum age of PL13 or older, reduced the odds to be culled by 94.2% (significant, p<0.02), compared to grow-out ponds stocked

		older							with PLs younger than a maximum age than PL13. Stocking grow-out ponds with older PLs had an apparent protective effect on survival.
Stocking - Husbandry	PL Age at Stocking	Min age PL10 or older	↓	6	0.07	0.01	0.34	0.001	Stocking grow-out ponds with PLs of a minimum age of PL10 or older, reduced the odds to be culled by 93.5% (significant, p<0.002), compared to grow-out ponds stocked with PLs younger than a minimum age of PL10. Stocking grow-out ponds with older PLs had an apparent protective effect on survival.
Post Stocking - Water Quality	Salinity during the First 6 weeks Post Stocking	Min ≥ 36 ppt	↓	6	0.01	0.00	0.09	<0.001	Grow-out ponds with a minimum salinity of 36ppt or more during the first 6 weeks post stocking, had reduced the odds to be culled by 98.6% (significant, p<0.002), compared to grow-out ponds with a minimum salinity below 36 ppt. Increased post stocking minimum salinity had an apparent protective effect on survival.
Stocking - Husbandry	Stocking Density	70 PL per sqm	↓	6	0.10	0.02	0.53	0.007	Stocking grow-out ponds with PLs at a density of 70 PL per square metre or higher, reduced the odds to be culled by 90.2% (significant, p<0.01), compared to grow-out ponds stocked with PLs at a density less than 70 PL per square metre. Stocking grow-out ponds with a higher density of PL had an apparent protective effect on survival.
Post Stocking - Water Quality	Total Ammonia Nitrogen (TAN) during the First 30 days Post Stocking	Av ≥ 0.5 mg/L	↓	6	0.05	0.01	0.42	0.006	Grow-out ponds with an average total ammonia nitrogen of 0.5 mg/L or more during the first 30 days post stocking, had reduced the odds to be culled by 95.3% (significant, p<0.01), compared to grow-out ponds an average total ammonia nitrogen below 0.5 mg/L. Increased post stocking average total ammonia nitrogen had an apparent protective effect on survival.
Post Stocking - Water Quality	Total Ammonia Nitrogen (TAN) during the First 30 days Post Stocking	Max ≥ 1.5 mg/L	↓	6	0.12	0.02	0.65	0.014	Grow-out ponds with a maximum total ammonia nitrogen of 1.5 mg/L or more during the first 30 days post stocking, had reduced the odds to be culled by 88.0% (significant, p<0.02), compared to grow-out ponds a maximum total ammonia nitrogen below 1.5 mg/L. Increased post stocking average total ammonia nitrogen had an apparent protective effect on survival.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Max ≥ 31 °C	↑	6	5.10	1.42	18.31	0.013	The odds of a grow-out pond that had a maximum water temperature of 31°C or more during the post stocking period (30 days), to be culled, was 5.10 times greater (significant, p<0.05) than the odds of a grow-out pond that maximum water temperature less than 31°C. Increased maximum water temperature in the post stocking period had an apparent detrimental effect on grow-out survival.
Acclimation - Water Quality	Dissolved Oxygen (DO) during acclimation (3 hours)	Min ≥ 8 mg/L	↑	6	11.14	1.22	102.03	0.033	The odds of a grow-out pond that had a maximum water temperature of 31°C or more during the post stocking period (30 days), to be culled, was 5.10 times greater (significant, p<0.05) than the odds of a grow-out pond that maximum water temperature less than 31°C. Increased maximum water temperature in the post stocking period had an apparent detrimental effect on grow-out survival.
Hatchery - Bacteria/ Toxin	zon occludens toxin gene CT value	Av CT ≥ 34	↑	6	5.33	1.19	23.83	0.028	The odds of a grow-out pond that had an average zon occludens toxin gene CT value of 34 or higher from routine hatchery sampling, to be culled, was 5.33 times greater than the odds of a grow-out pond that had an average zon occludens toxin gene CT value of less than 34. Higher average zon occludens toxin gene CT value (i.e. lower toxin gene quantity) in hatchery PLs subsequently stocked into grow-out ponds had an apparent detrimental effect on survival.
Acclimation - Water Quality	Dissolved Oxygen (DO) during Acclimation (3	Max ≥ 10 mg/L	↔	5	-	-	-	>0.05	Grow-out ponds that had an acclimation (3 hours) maximum DO of 10 mg/L or higher did not significantly (p>0.05) associate with being culled.

	hours)								
Hatchery - Chemicals/ Therapeutics	Oxytetracycline (Immersion) treatment	One or more	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PLs treated with Oxytetracycline 1 or more times during larval rearing did not significantly (p>0.05) associate with being culled. All (8/8) Grow-out populations from hatchery tanks with no Oxytetracycline exposure predicted survival 100%
Acclimation - Water Quality	pH during Acclimation (3 hours)	Max ≥ 8.7 units	↔	5	-	-	-	>0.05	Grow-out ponds that had an acclimation (3 hours) maximum pH of 8.7 or higher did not significantly (p>0.05) associate with being culled.
Acclimation - Water Quality	Water Temperature during Acclimation (3 hours)	Max ≥ 27 °C	↔	5	-	-	-	>0.05	Grow-out ponds that had an acclimation (3 hours) maximum water temperature of 27°C or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Max daily change ≥ 3 °C	↔	5	-	-	-	>0.05	Grow-out ponds that over the post stocking period (30 days) had a maximum daily water temperature of 3°C or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Min ≥ 24 °C	↔	5	-	-	-	>0.05	Grow-out ponds that over the post stocking period (30 days) had a minimum daily water temperature of 24°C or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Cyanobacteria score during the First 30 days Post Stocking	Av score ≥ 8	↔	5	-	-	-	>0.05	Grow-out ponds with an average cyanobacteria score of 8 or higher (0=absent; 5=low; 10=medium; 15=high) over the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Cyanobacteria sp. during the First 30 days Post Stocking	Max score ≥ 10	↔	5	-	-	-	>0.05	Grow-out ponds that during the post stocking period (30 days) had a maximum cyanobacteria score of 10 (0=absent; 5=low; 10=medium; 15=high) or higher over did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Max ≥ 13 mg/L	↔	5	-	-	-	>0.05	Grow-out ponds that during the post stocking period (30 days) had a maximum daily DO of 13 mg/L or higher did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Daily Av. Change ≥ 5 mg/L	↔	5	-	-	-	>0.05	Grow-out ponds with an average daily DO change of 5 mg/L or higher during the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Daily Av. ≥ 7.5 mg/L	↔	5	-	-	-	>0.05	Grow-out ponds with an average daily DO of 7.5 mg/L or higher during the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Hatchery - Chemicals/ Therapeutics	Erythromycin (Immersion) treatment	One or more	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PLs treated with Erythromycin during larval rearing did not significantly (p>0.05) associate with being culled. There were no Grow-out populations without exposure to Erythromycin during larval rearing, which limited the comparative analysis.
Hatchery - Performance	Hatchery (Larval Rearing) Survival	Av. Percent	↔	5	-	-	-	>0.05	Grow-out ponds stocked in PLs from hatchery tanks that had an average (larval rearing) survival of greater than or equal to 10% (or 20% or 30% or 40%) were not significantly (p>0.05) associated with being culled. Note that only hatchery (larval rearing) tanks that survived could be analysed (Survival bias).
Hatchery - Performance	Hatchery (Larval Rearing) Survival	Max Percent	↔	5	-	-	-	>0.05	Grow-out ponds stocked in PLs from hatchery tanks that had a maximum (larval rearing) survival of greater than or equal to 30% (or 40% or 50% or 60%) were not significantly (p>0.05) associated with being culled. Note that only hatchery (larval rearing) tanks that

									survived could be analysed (Survival bias).
Hatchery - Performance	Hatchery (Larval Rearing) Survival	Min Percent	↔	5	-	-	-	>0.05	Grow-out ponds stocked in PLs from hatchery tanks that had a minimum (larval rearing) survival of greater than or equal to 10% (or 20% or 30% or 40%) were not significantly (p>0.05) associated with being culled. Note that only hatchery (larval rearing) tanks that survived could be analysed (Survival bias).
Stocking - Husbandry	Hatchery Tanks used for pond stocking	5 or more	↔	5	-	-	-	>0.05	Grow-out ponds that were stocked with 5 or more hatchery tanks did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Mixed Dinoflagellates during the First 30 days Post Stocking	Max score ≥ 5	↔	5	-	-	-	>0.05	Grow-out ponds that had a maximum mixed dinoflagellate score of 5 or higher (0=absent; 5=low; 10=medium; 15=high) over the first 30 days post stocking did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Daily Av. ≥ 8.3 units	↔	5	-	-	-	>0.05	Grow-out ponds with an average daily pH of 8.3 or higher over the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Min. ≥ 8 units	↔	5	-	-	-	>0.05	Grow-out ponds that over the post stocking period (30 days) had a minimum daily pH of 8.0 or higher did not significantly (p>0.05) associate with being culled.
Stocking - Husbandry	PL Age at Stocking	Av age PL11 or older	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PLs of an average age of PL11 or older did not significantly (p>0.05) associate with being culled.
Stocking - Health Assessment	PL Health Score prior to stocking	Av score ≥ 90	↔	5	-	-	-	>0.05	Grow-out ponds that had an average health score of 90 or higher from pre-stocking PLs did not significantly (p>0.05) associate with being culled.
Stocking - Health Assessment	PL Stress Test Score prior to stocking	Av score ≥ 6	↔	5	-	-	-	>0.05	Grow-out ponds that had an average stress score of 6 or higher from pre-stocking PLs did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	RtX toxin gene CT value	Av CT ≥ 32	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PL that had an average RtX toxin gene CT value of 32 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	RtX toxin gene CT value	Yes	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PL that had an RtX toxin gene CT detected, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	RtX toxin gene CT value	Min CT ≥ 3	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PL that had a minimum RtX toxin gene CT value of 30 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Salinity during the First 6 weeks Post Stocking	Max ≥ 41 ppt	↔	5	-	-	-	>0.05	Grow-out ponds that over the post stocking period (6 weeks) had a maximum salinity of 41 ppt or higher did not significantly (p>0.05) associate with being culled.
Pond Preparation - Husbandry	Stocking PLs into grow-out ponds over an extended duration	Days	↔	5	-	-	-	>0.05	Grow-out ponds stocked PLs over an extended period of 6, 7, or 8 or more days did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Av daily ≥27.5°C	↔	5	-	-	-	>0.05	Grow-out ponds with an average water temperature of 27.5°C or higher over the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Av daily change ≥2°C	↔	5	-	-	-	>0.05	Grow-out ponds with an average daily water temperature change of 2°C or higher over the post stocking period (30 days) did not significantly (p>0.05) associate with being culled.

Hatchery - Bacteria/ Toxin	YAFO toxin gene	Yes	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PL that had an YaFO toxin gene CT detected, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	YAFO toxin gene CT value	Av CT ≥ 32	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PL that had an average YaFO toxin gene CT value of 32 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	YAFO toxin gene CT value	Min CT ≥ 30	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PL that had a minimum YaFO toxin gene CT value of 30 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	zon occludens toxin gene	Yes	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PL that had a zon occludens toxin gene detected, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Bacteria/ Toxin	zon occludens toxin gene CT value	Min CT ≥ 34	↔	5	-	-	-	>0.05	Grow-out ponds stocked with PL that had a minimum zon occludens toxin gene CT value of 34 or higher, pre-stocking, did not significantly (p>0.05) associate with being culled.
Hatchery - Chemicals/ Therapeutics	Oxytetracycline (Immersion) treatment	Three or more	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have received more Oxytetracycline treatments in the hatchery (outside the range of standard error of the mean).
Acclimation - Water Quality	Water Temperature during Acclimation (3 hours)	Max >27.7°C	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely experience a higher maximum temperature during acclimation to grow-out ponds (outside the range of standard error of the mean).
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Max >30°C	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a higher maximum daily temperature during the post stocking period (outside the range of standard error of the mean).
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Av daily change > 4 mg/L	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a higher average daily dissolved oxygen change during the post stocking period (outside the range of standard error of the mean).
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Min > 4.3 mg/L	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a lower minimum dissolved oxygen level during the post stocking period (outside the range of standard error of the mean).
Post Stocking - Water Quality	Gymnodinium sp during the First 30 days Post Stocking	Av score >1	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a higher average gymnodinium algae score over routine monitoring during the post stocking period (outside the range of standard error of the mean). Score: 0=absent; 5=low; 10=medium; 15=high.
Post Stocking - Water Quality	Mixed Nitzschia sp. during the First 30 days Post Stocking	Av score >2	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a higher average Nitzschia algae score over routine monitoring during the post stocking period (outside the range of standard error of the mean). Score: 0=absent; 5=low; 10=medium; 15=high.
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Av daily change >0.2 unit	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a higher average daily pH change during the post stocking period (outside the range of standard error of the mean).
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Av daily >8.3 unit	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to experience a higher average daily pH during the post stocking period (outside the range of standard error of the mean).
Post Stocking -	pH during the First	Max >	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to experience a higher maximum

Water Quality	30 days Post Stocking	9.0 units							daily pH during the post stocking period (outside the range of standard error of the mean).
Hatchery - Bacteria/ Toxin	RtX toxin gene CT value	Av CT > 32	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a higher RtX toxin gene CT value in pre-stocking hatchery testing (outside the range of standard error of the mean).
Post Stocking - Water Quality	Salinity during the First 6 weeks Post Stocking	Av Max change > 5.0 ppt	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to experience a higher maximum salinity change during the post stocking period (outside the range of standard error of the mean).
Pond Preparation - Husbandry	Stocking PLs into grow-out ponds over an extended duration	Av over four days	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to be stocked over a higher number of days (outside the range of standard error of the mean).
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Av daily >27.0°C	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a higher average daily temperature during the post stocking period (outside the range of standard error of the mean).
Hatchery - Bacteria/ Toxin	YAFO toxin gene CT value	Av CT > 32.5	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a higher YaFO toxin gene CT value in pre-stocking hatchery testing (outside the range of standard error of the mean).
Hatchery - Bacteria/ Toxin	zon occludens toxin gene	Yes	↑	4	-	-	-	-	Culled grow-out ponds were on average more likely to have zon occludens toxin gene detected in pre-stocking hatchery testing (within the range of standard error of the mean).
Pond Preparation - Husbandry	Days Between completion of initial pond filling and Stocking (Number)	Av Over nine days	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a shorter period between filling with water and stocking of PLs (outside the range of standard error of the mean).
Pond Preparation - Husbandry	Days Between initial Pond Fertilisation and Stocking	Av Over eight days	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a shorter period between fertilisation and stocking of PLs (outside the range of standard error of the mean).
Hatchery - Chemicals/ Therapeutics	Erythromycin (Immersion) treatment	Av over 1.5 times	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to have received less Erythromycin treatments in the hatchery (outside the range of standard error of the mean).
Post Stocking - Water Quality	Mixed Dinoflagellates sp. during the First 30 days Post Stocking	Av score > 3	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a lower average mixed dinoflagellate algae score over routine monitoring during the post stocking period (outside the range of standard error of the mean). Score: 0=absent; 5=low; 10=medium; 15=high.
Stocking - Husbandry	PL Age at Stocking	Av age PL12 or older	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to be stocked with younger PLs (outside the range of standard error of the mean).
Post Stocking - Water Quality	Salinity during the First 6 weeks Post Stocking	Av Min > 35 ppt	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a lower minimum salinity during the post stocking period (outside the range of standard error of the mean).
Stocking - Husbandry	Stocking Density	Av 60PL per sqm	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to be stocked with a lower density of PLs (outside the range of standard error of the mean).
Post Stocking - Water Quality	Total Ammonia Nitrogen (TAN) during the First 30 days Post Stocking	Av >0.30 mg/L	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to experience a lower average total ammonia nitrogen (TAN) during the post stocking period (outside the range of standard error of the mean).

Post Stocking - Water Quality	Total Ammonia Nitrogen (TAN) during the First 30 days Post Stocking	Av Max >0.80 mg/L	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to experience a lower maximum total ammonia nitrogen (TAN) during the post stocking period (outside the range of standard error of the mean).
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Av daily change >1.90°C	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a lower average daily temperature change during the post stocking period (outside the range of standard error of the mean).
Hatchery - Bacteria/ Toxin	zon occludens toxin gene CT value	Av CT >32	↓	4	-	-	-	-	Culled grow-out ponds were on average more likely to have a lower zon occludens toxin gene CT value in pre-stocking hatchery testing (outside the range of standard error of the mean).
Acclimation - Water Quality	Dissolved Oxygen during Acclimation (3 hours)	Max	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower maximum dissolved oxygen level during the acclimation period (within the range of standard error of the mean).
Acclimation - Water Quality	pH during Acclimation (3 hours)	Max	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower maximum pH during acclimation to grow-out ponds (within the range of standard error of the mean).
Post Stocking - Water Quality	Water Temperature during the First 30 days Post Stocking	Max daily change	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower maximum daily water temperature change during the post stocking period (within the range of standard error of the mean).
Post Stocking - Water Quality	Cyanobacteria score during the First 30 days Post Stocking	score	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower average cyanobacteria algae score over routine monitoring during the post stocking period (within the range of standard error of the mean). Score: 0=absent; 5=low; 10=medium; 15=high.
Post Stocking - Water Quality	Dissolved Oxygen (DO) during the First 30 days Post Stocking	Max	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower maximum daily dissolved oxygen levels during the post stocking period (within the range of standard error of the mean).
Acclimation - Water Quality	Dissolved Oxygen (DO, mg/L) during acclimation (3 hours)	Min	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower minimum dissolved oxygen level during the acclimation period (within the range of standard error of the mean).
Post Stocking - Water Quality	Dissolved Oxygen (DO, mg/L) during the First 30 days Post Stocking	Av daily	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower maximum daily water temperature change during acclimation to grow-out ponds (within the range of standard error of the mean).
Hatchery - Performance	Hatchery (Larval Rearing) Survival	precent	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower hatchery survival (within the range of standard error of the mean).
Stocking - Husbandry	Hatchery Tanks used for pond stocking	number	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower number of contributing hatchery tanks to pond stocking (within the range of standard error of the mean).
Post Stocking - Water Quality	pH during the First 30 days Post Stocking	Min	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower minimum pH during the post stocking period (within the range of standard error of the mean).
Stocking - Health Assessment	PL Health Score prior to stocking	percent score	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower stress test score prior to stocking (within the range of standard error of the mean).
Stocking - Health	PL Health Score prior to stocking	percent score	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower stress test score prior to stocking (within the range of standard error of the mean).

Assessment									
Hatchery - Bacteria/ Toxin	RtX toxin gene CT value	Yes	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have RtX toxin gene detected in pre-stocking hatchery testing (within the range of standard error of the mean).
Post Stocking - Water Quality	Salinity (ppt) during the First 6 weeks Post Stocking	Max	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have a higher or lower maximum salinity during the post stocking period (within the range of standard error of the mean).
Hatchery - Bacteria/ Toxin	YAFO toxin gene	Yes	↔	3	-	-	-	-	Culled grow-out ponds were not more likely to have YaFO toxin gene detected in pre-stocking hatchery testing (within the range of standard error of the mean).
Post Stocking - Water Quality	Neonicotinoids	Variable	↔	1	-	-	-	-	Neonicotinoids did not appear to have an effect on Grow-out survival. 7 compounds screened (See Appendix 5 for more details)
Post Stocking - Water Quality	Personal care products (PPCPs)	Variable	↔	1	-	-	-	-	Personal care products (PPCPs) did not appear to have an effect on Grow-out survival. 81 compounds screened (See Appendix 5 for more details)
Grow-out - Viruses	Gill-associated virus (GAV)	Yes	↔	1	-	-	-	-	Submission Reference: P18-06072F (Pond 4, n=10); P18-06074F (pond 6, n=9); P18-06077F (pond 45, n=5); P18-06075F (Pond 7, n=10); P18-06076F (Pond 14, n=10); P18-06073F (pond 5, n=5); P18-05992F (n/a, n=3). Collection Date: 20/12/18 (all ex. P18-05992F = 12/12/18). ^ 1 additional suspect positive
Grow-out - Bacteria	Vibrio harveyi	Yes	↔	1	-	-	-	-	Submission Reference: P19-001070F (Pond 5, n=6). Isolates in light pure to moderate mixed growth. Collection date: 10/01/19
Grow-out - Bacteria	Vibrio parahaemolyticus	Yes	↔	1	-	-	-	-	Submission Reference: P19-001070F (Pond 5, n=6). Isolates in light pure to moderate mixed growth. Collection date: 10/01/19
Grow-out - Bacteria	Photobacterium damselae	Yes	↔	1	-	-	-	-	Submission Reference: P19-001070F (Pond 5, n=6). Isolates in light pure to moderate mixed growth. Collection date: 10/01/19
Grow-out - Viruses	Infectious hypodermal and hematopoietic necrosis (IHHNV)	Yes	↔	**0**	-	-	-	-	Submission Reference: P18-06072F (Pond 4, n=10); P18-06074F (pond 6, n=9); P18-06077F (pond 45, n=5); P18-06075F (Pond 7, n=10); P18-06076F (Pond 14, n=10); P18-06073F (pond 5, n=5); P18-05992F (n/a, n=3). Collection Date: 20/12/18 (all ex. P18-05992F = 12/12/18). ^ 3 suspect positives
Grow-out - Bacteria/ Toxins	Pir A / Pir B	Yes	↔	**0**	-	-	-	-	Submission Reference: P18-06072F (Pond 4, n=10); P18-06074F (pond 6, n=9); P18-06077F (pond 45, n=5); P18-06075F (Pond 7, n=10); P18-06076F (Pond 14, n=10); P18-06073F (pond 5, n=5); P18-05992F (n/a, n=3). P19-001070F (Pond 5, n=6). Collection Dates: 20/12/18 (all ex. P18-05992F - 12/12/18; P19-001070F -10/01/19).
Grow-out - Viruses	Taura Syndrome Virus (TSV)	Yes	↔	**0**	-	-	-	-	Submission Reference: P18-06072F (Pond 4, n=10); P18-06074F (pond 6, n=9); P18-06077F (pond 45, n=5); P18-06075F (Pond 7, n=10); P18-06076F (Pond 14, n=10); P18-06073F (pond 5, n=5); P18-05992F (n/a, n=3). Collection Date: 20/12/18 (all ex. P18-05992F = 12/12/18).
Grow-out - Viruses	White Spot Syndrome Virus (WSSV)	Yes	↔	**0**	-	-	-	-	Submission Reference: P18-06072F (Pond 4, n=10); P18-06074F (pond 6, n=9); P18-06077F (pond 45, n=5); P18-06075F (Pond 7, n=10); P18-06076F (Pond 14, n=10); P18-06073F (pond 5, n=5); P18-05992F (n/a, n=3). Collection Date: 20/12/18 (all ex. P18-05992F = 12/12/18).
Grow-out - Viruses	Yellow head virus (Genotype 1)	Yes	↔	**0**	-	-	-	-	Submission Reference: P18-06072F (Pond 4, n=10); P18-06074F (pond 6, n=9); P18-06077F (pond 45, n=5); P18-06075F (Pond 7, n=10); P18-06076F (Pond 14, n=10); P18-06073F (pond 5, n=5); P18-05992F (n/a, n=3). Collection Date: 20/12/18 (all ex. P18-05992F = 12/12/18).
Grow-out - Viruses	Yellow head virus (Genotype 7)	Yes	↔	**0**	-	-	-	-	Submission Reference: P18-06072F (Pond 4, n=10); P18-06074F (pond 6, n=9); P18-06077F (pond 45, n=5); P18-06075F (Pond 7, n=10); P18-06076F (Pond 14, n=10); P18-06073F (pond 5, n=5); P18-05992F (n/a, n=3). Collection Date: 20/12/18 (all ex.

									P18-05992F = 12/12/18).

Appendix 4 : References

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