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BONAMIOSIS IN FARMED NATIVE OYSTERS (*Ostrea angasi*)

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FRDC Project No **2015/001**

ISBN 978-1-76090-307-7

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Project No. 2015/001

2019

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In submitting this report, the researcher has NOT agreed to FRDC publishing this material in its edited form. Please note discussion

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Acknowledgments

The authors thank: Joanne Slater, Stacey Valdeter and David Cummins for their assistance in preparation of negative Native Oyster material and the training of Jessica Buss from SARDI at CSIRO Australian Animal Health Laboratory – Fish Diseases Laboratory (AFDL).

We would also like to acknowledge the assistance of staff at AgriBio, Bundoora Victoria for the preparation of histological slides, Dr John Humphrey for assisting with the reading of all histopathological slides and staff at CSIRO AFDL for assisting with the preparation and qPCR analysis of both trial and surveillance samples. Importantly, we wish to acknowledge the assistance of Victorian flat oyster growers, particularly Peter Bold, Peter Lillie and Steve Cooper.

Abbreviations

AAHL	Australian Animal Health Laboratory
aqCCEAD	Aquatic Consultative Committee for Emergency Animal Disease
AFDL	AAHL Fish Diseases Laboratory
BLAST	Basic Local Alignment Search Tool
BLASTn	Basic Local Alignment Search Tool (for) nucleotide sequences
BLASTp	Basic Local Alignment Search Tool (for) protein sequences
cDNA	Complementary DNA
CI	Credible Intervals
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C_T	Cycle threshold
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DSe	Diagnostic sensitivity
DSp	Diagnostic specificity
EST	Expressed sequence tag
FRDC	Fisheries Research and Development Corporation
GLM	Generalised linear mixed model
HMM	Hidden Markov Models
HR	Hazard ratios
ISH	In situ hybridisation
ITS	Internal transcribed spacer
JAGS	Just Another Gibbs Sampler
LCI	Lower Confidence Interval
LCM	Latent Class Model
LR	Likelihood ratio

LSU	Large-subunit
MCMC	Markov Chain Monte Carlo
mS/cm	Milli Siemens per centimetre
NaOCL	Sodium hypochlorite
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NPV	Negative predictive value
Nr	Non-redundant
OIE	World Organisation for Animal Health (Office International des Épizooties)
OR	Odds Ratio
PCI	Posterior Credible Intervals
PCR	Polymerase chain reaction
PPM	parts per million
PPV	Positive Predictive Value
PV	Predictive Value
QAC	Quaternary ammonium compound
qPCR	Quantitative (real-time) PCR
ROC	Receiver Operating Curve
rRNA	Ribosomal ribonucleic acid
SAABC	South Australian Aquatic Biosecurity Centre
SARDI	South Australian Research and Development Institute
Se	Sensitivity
Sp	Specificity
SSU	Small-subunit
UCI	Upper Confidence Interval

1. Executive Summary

What the report is about

This project was a collaborative study across three research organisations: Agriculture Victoria, CSIRO (Victoria) and the South Australian Research and Development Institute (SARDI). The overall aim of this collaborative project was to better understand many aspects of infection with the parasite *Bonamia exitiosa* in Native Oysters (*Ostrea angasi*). The genesis of this project was the detection of clinical bonamiosis in farmed Native Oysters in Port Phillip Bay, Victoria in 2015. At the time the species of this parasite was unknown, and it had caused extensive mortalities (presumed over 80%) in stock on one farm.

CSIRO demonstrated that current diagnostic PCR assays are sufficient and effective in the detection and identification of *Bonamia* species from farmed *O. angasi* in southern Australia. The species of *Bonamia* parasite present in farmed *O. angasi* between 2013 and 2017 in Victoria, New South Wales and South Australia was confirmed to be *B. exitiosa*. Next generation sequencing (NGS) and bioinformatic analysis of nucleotides extracted from *B. exitiosa* infected and uninfected *O. angasi* facilitated assembly of the first draft genome of a member of the family Haplosporidiidae, *B. exitiosa.* For the first time a draft genome was also assembled for *O. angasi* as a result of the sequencing strategy undertaken to identify the *B. exitiosa* genome. Unexpectedly, a near complete genome was also assembled for an Epsilon proteobacterium, *Poseidonibacter* from *O. angasi* tissues infected with *B. exitiosa*. This organism may have been an adventitious discovery or potentially proliferated in flat oysters weakened by *B. exitiosa* infection.

In Victoria, 4 individual tank and field trials were undertaken between 2016 and 2018. All trials were designed to investigate proposed risk factors for the development of clinical bonamiosis under normal farming or controlled laboratory conditions. The tank trials utilised presumed sub-clinically infected and healthy oysters and subjected them to stressors such as heat, starvation and turbulence. Further tank trials examined oyster origin and size as risk factors. The field trials utilised existing farm sites and practices to investigate a number of proposed risk factors of interest to the farmers. The field trials were conducted on a known clinically infected farm and examined the risk factors including basket density, basket depth in the water column, oyster size and level of fouling. Concurrently, the project validated the diagnostic performance of the *Bonamia* sp. qPCR and established the optimal epidemiological qPCR cycle threshold (C_T) value to differentiate between a positive and negative result.

SARDI undertook a range of trials in both the field and under laboratory conditions. Oysters were tested from 3 farms to assess diagnostic sensitivity (DSe) and specificity (DSp) of heart smears, histopathology and qPCR individually or in combination, and to assess prevalence. Tank trials were utilised to develop a cohabitation infection model using uninfected hatchery-reared recipient animals and infected donor animals from farms to better understand infection dynamics. The Pacific oyster, *Crassostrea gigas*, was shown to be susceptible to *Bonamia exitiosa* by cohabitation in the laboratory. A decontamination trial was undertaken in the laboratory using heavily infected Native Oysters to assess processes for decontaminating equipment that may have been exposed to *Bonamia exitiosa.* These

approaches were combined in a field trial where *O. angasi* were deployed at Cowell, Coffin Bay and Streaky Bay to examine the prevalence of *B. exitiosa* over time related to measured environmental parameters and growth rates. SARDI also assessed 3 different diagnostic tests for detecting *Bonamia* sp: heart smears, histology and qPCR. In this work the effect of combining tests to maximise overall diagnostic performance was also investigated.

Background

There has been a history of mass mortalities of the Native Oyster in Victoria since the early 1990s and more recently following attempts to farm the oyster with both basket and rope culture. The cause of these mortalities was a parasite belonging to the genus *Bonamia.* It is accepted that this parasite is endemic in parts of Australia and yet little is known about it. Interest in Native Oyster culture remains strong in response to the prospect of good markets and for the purposes of reef restoration projects. This project aimed to develop a greater understanding of the *Bonamia* organism that is present in Native Oyster populations in south eastern Australia including infection dynamics, diagnostics, epidemiology, and management.

Aims/objectives

1) *Obtain nucleic acid sequence and compare with other, described* Bonamia *sp. and determine their taxonomic relationship and ensure that available diagnostic tools are suitable.*

2) *Improve understanding of bonamiosis infestations in Native Oysters including the determination, under controlled conditions, of the stressors that induce clinical disease in sub - clinically infected oysters.*

3) *Develop a biosecurity plan and farm management practices to manage the risk of infestation and the mitigation of clinical infection with* Bonamia *sp.*

Methodology

This project was conducted under laboratory and field conditions at sites in Victoria and South Australia. A range of diagnostic methodologies were used including qPCR, histopathology, heart smears and next generation sequencing to determine both oyster health status with relation to *Bonamia* and aid in the comparison and validation of tests. Field trials were conducted as part of this project and involved more advanced diagnostics to determine health status of oysters at field sites or simpler observations such as determining mortality state and size or weight.

Results/key findings

CSIRO

More than a billion DNA sequences from *B. exitiosa* infected and uninfected *O. angasi* tissue were analysed to generate the first assembled *B. exitiosa* draft genome. The *B. exitiosa* genome was 9.5 Mb with a low GC content and encoded a predicted 8,007 genes. The protein function of 44.2% of the genes were determined and predicted to be predominately involved in molecule transport, proteolysis and oxidation-reduction processes. The most closely related genes were found to be from *Plasmodiophora brassicae*, classified in the same supergroup of eukaryotes as *Bonamia* (Rhizaria) and the same phylum (Cercozoa).

During the approach undertaken to assemble the *B. exitiosa* genome, an *O. angasi* draft genome was also assembled. The genome was estimated to be 85% complete, 672 Mb in size and encoding 72,073 genes, with similarities in size and gene number to other assembled oyster genomes. Unexpectedly, a bacterial genome was also assembled and identified to be from the *Poseidonibacter* genus. The bacterial genome was 3.5 Mb, contained 3,449 genes and was estimated to be 95% complete.

Agriculture Victoria

The first field trial found the only significant risk factor was size of oyster: smaller oysters had a 10% higher mortality rate than larger oysters. In the second field trial the risk factors investigated were basket density and level of fouling, but this trial did not yield any significant findings. In both trials the mortality rates were over 30%.

In the tanks trials, for the end point "oyster death", the risk factors starvation alone and several combinations including starvation, hot water and tumbling yielded significantly higher mortality rates. When the data from this trial was reanalysed using the newly established qPCR C_T value cut-point of ≤34.5 the risk factors of heating alone and a combination of heat/starved and tumbled resulted in an increased risk of death. In the second tank trial, the factors oyster size and origin were studied under normal and "stressed" conditions (heated water and starvation). Oysters were selected from the clinically infected farm and a farm with known prevalence of infection but no clinical disease. The case fatality rate and likelihood of death was found to be higher in the farm that had not experienced previous clinical disease. Survival analysis similarly, confirmed that *Bonamia* infected oysters from the non-clinical farm died 3.76 times the rate of those from the clinically infected farm. This suggests either genetic differences conferring a benefit or some form of resistance developing across the two farms.

Using an epidemiological approach, the diagnostic sensitivity and specificity of the *Bonamia* sp. qPCR were calculated for increasing C_T value epidemiological cut points from ≤25 to ≤40, with an optimal cut point identified at ≤34.5 (DSp: 0.92; 95% PCI: 0.76, 0.99; and DSe: 0.94; 95% PCI: 0.85, 0.99). The diagnostic sensitivity of qPCR was significantly higher compared to histopathological examination, while the diagnostic specificity of both tests was very high (>0.92).

SARDI

SARDI found using a Bayesian Latent Class Model to assess diagnostic sensitivity (DSe) and specificity (DSp) that histology was the best individual test (DSe 0.76, DSp 0.93) compared to quantitative polymerase chain reaction (qPCR) (DSe 0.69, DSp 0.93) and heart smear (DSe 0.61, DSp 0.60). Histology combined with qPCR and defining a positive from either test as an infected case maximised test performance (DSe 0.91, DSp 0.88). Prevalence was higher at two farms in a high-density oyster growing region than at a farm cultivating oysters at lower density. SARDI infection dynamics studies showed that infection occurs, abundance builds, and mortality due to clinical *B. exitiosa* infection occur rapidly in <2 g *O. angasi*. Host death is not required for transmission. These experiments showed that cohabitation provides an informative way to mimic natural infection by *B. exitiosa* and study *B. exitiosa* transmission. Infection occurs rapidly, with *B. exitiosa* prevalence, intensity, and mortalities among recipient oysters increasing with continued exposure. These trials also demonstrated that Pacific oysters (*Crassostrea gigas*) are susceptible to *B. exitiosa* but did not develop clinical disease. Field trials at 3 locations in South Australia resulted in oysters becoming infected with *B. exitiosa* within 3 months with typical, mostly low abundance type infections. None of the environmental parameters measured were correlated with infection intensity or prevalence, and the environmental drivers of *B. exitiosa* infections in South Australia remain unclear. Prevalence at all sites increased over time, but intensity remained low. The decontamination trial found that the quarternary ammonium detergent used was not suitable for decontaminating equipment exposed to *B. exitiosa* but was suitable for cleaning. Hypochlorite however, at 4% active chlorine for 10 minutes, reliably inactivated 100% of *B. exitiosa* cells, and provides a useful basis for decontaminating equipment.

The different results for diagnostic sensitivity and specificity for the *Bonamia* sp. qPCR may be due to the different approaches used by the institutes and differing prevalence between sites. SARDI calculated parameters based on an analytical cut-point versus the epidemiological cut-off used by Agriculture Victoria. Further conditional dependence (covariance) between diagnostic tests was allowed in SARDIs methodology, whilst Agriculture Victoria assumed conditional independence within the latent class analysis.

Implications for relevant stakeholders

The work undertaken by Agriculture Victoria suggests that the application of an optimal epidemiological C_T value is appropriate for enzootic organisms where the farmer is attempting to minimize the economic impact of a pathogen through limiting the number of false positive and false negative diagnoses.

SARDI utilised an analytical approach to establishing the optimal C_T value that maximises DSe where new areas are being surveyed for *B. exitiosa*, but may assign some samples as positive that other approaches would classify as negative. An epidemiological approach maximises the certainty that positive results are accurate but assigns some samples as negative that other approaches would assign as positive. This would be beneficial in determining prevalence in endemic areas.

The *Bonamia* sp. qPCR is genus-specific; development of a new assay that detects *B. exitiosa* but not other *Bonamia* spp. is a priority. Species-specific assays for *B. exitiosa* and *B. ostreae* are in development at NIWA, New Zealand, and SARDI has provided some input to test development.

Bonamia surveillance should be undertaken by the *Bonamia* sp. qPCR as a screening test which is faster, cheaper and may have a higher level of diagnostic sensitivity than histopathology. The diagnostic sensitivity and specificity of heart smears is lower than other test types however the low cost of conducting this test makes it an attractive option where other confirmatory tests are available.

This project demonstrated that Pacific oysters (*Crassostrea gigas*) are susceptible to *B. exitiosa*, and their role in field infections of Native Oysters requires clarification. The current OIE manual on *B. exitiosa* requires updating with respect to susceptible species.

Stressors such as water warmed beyond the ideal range can result in the development of clinical bonamiosis in subclinically infected Native Oysters. There may be implications for both farmed and wild stock in environments affected by climate change.

The different prevalence and intensity of infection between different sites in South Australia support that *B. exitiosa* infections rapidly build on farm in South Australia and highlights which regions are appropriate for industry expansion.

Hypochlorite may be a useful chemical in terms of decontaminating equipment to reduce the risk of *Bonamia* transmission on fomites.

Keywords

Bonamia exitiosa, Native Oysters (*Ostrea angasi*), *Bonamia* sp. qPCR; epidemiology; decontamination.

2. Introduction

Native Oysters (*Ostrea angasi*) have been an important food source for indigenous people in Australia for millennia, and post colonisation extensive wild harvest depleted supplies from the mid - 1800s in bays and inlets around Victoria, South Australia, and other parts of the country. There have been several attempts to farm this species in Port Phillip Bay, Victoria and other locations. In Victoria such attempts were devastated by a parasite belonging to the genus *Bonamia* in 1991 that resulted in the closure of Native Oyster farms. *Bonamia* was further identified in Georges Bay, Tasmania (1992) and in Albany, Western Australia in 1993 (Hine & Jones, 1994).

Infection with the haplosporidian *Bonamia* sp., has caused substantial negative impacts on ostreine oysters worldwide, including *Tiostrea chilensis*, *O. edulis*, *O. angasi*, *O. stentina* and *O. puelchana* (see Engelsma et al., 2014; Hine & Jones, 1994). *Bonamia* microcells infect phagocytic haemocytes which are the primary cellular immune defence for oysters (Hine, 1996). *Bonamia* blocks haemocyte production of oxidative radicals, which facilitates parasite multiplication, leading to a systemic infection with high parasite intensity, loss of condition and death (Corbeil, Handlinger & Crane, 2009). It is therefore important to evaluate the intensity of *Bonamia* infection and prevalence to assess oyster health, particularly for surveillance in new areas or populations.

Despite the early set back in farming attempts, interest in Native Oyster culture remains strong in south-east Australia in response to the prospect of good domestic and international markets. The role of wild *O. angasi* as ecosystem engineers has generated further interest in reef restoration (Gillies, Crawford & Hancock, 2017) and the risks that bonamiosis poses to current reef restoration projects needs to be considered. Further, diversification from the main farmed oyster species in Australia, the Pacific oyster (*Crassostrea gigas*) provides opportunities to safeguard against the effects of OsHV-1 microvariant that has caused production losses in Australian Pacific oyster culture since 2010.

Annual monitoring of Victorian Native Oyster farms by the *Bonamia* sp. quantitative polymerase chain reaction (qPCR) and histopathological examination began in 2011 when small scale farming of Native Oysters recommenced. Infection in apparently healthy oysters was detected a few years later and over time increased in prevalence, culminating in a clinical outbreak of bonamiosis on one site associated with high levels of mortalities (Bradley, pers comm). Sequencing undertaken determined that this outbreak was due to *B. exitiosa*. Apparent subclinical infection was detected by *Bonamia* sp. qPCR surveillance in wild Native Oysters in Port Phillip Bay and on some farm sites. However, this infection did not progress to clinical expression. Given the similar origin of source stock and the history of bonamiosis in Victorian waters, the question of why some farm sites progress to clinical disease where others remain healthy was of interest.

To investigate this issue, identification of risk factors that may trigger clinical disease/death due to *B. exitiosa* infection in Australian *O. angasi*, including source location, oyster size and water temperature, was undertaken. It is presumed that where these factors are known and can be manipulated, management to prevent the devastation of Native Oyster crops caused by Bonamiosis can be undertaken.

Minimal genomic information is currently available for the molluscan parasites within the family Haplosporidiidae. For instance, the taxonomic relationship between *Bonamia* species, such as *B. exitiosa* and *B. ostreae,* both of global significance for oyster health, are based on a small number of genes. This is due to no *Bonamia* genomes being publicly available for greater analysis. The discovery of the genome from the *Bonamia* parasite infecting Native oysters in southern Australia would provide unprecedented insight into its classification and characterisation.

The status and effects of *Bonamia* on South Australian Native Oysters was less clear at the start of this study than it was for Victorian Native Oysters. In South Australia this project assessed different test methodologies, particularly on high prevalence low intensity populations, used laboratory studies to understand infection dynamics and the susceptibility of the Pacific oyster (*Crassostrea gigas*) to *Bonamia* and assessment a range of decontamination regimes. A field study examined infection dynamics in three growing regions and examined environmental parameters and site as influences of infection.

Understanding disease risks is therefore important for assessing the viability of expansion of *O. angasi* aquaculture and restoration of wild populations.

3. CSIRO – AFDL

Assessment of the effectiveness of current diagnostic tools and the genome sequencing of *Bonamia exitiosa*

3.1 Summary

The identity of the *Bonamia* species present in farmed Australian flat oysters (*Ostrea angasi*) between 2013 and 2017 in Victoria, New South Wales and South Australia was confirmed with currently available diagnostic tools to be *Bonamia exitiosa*. A draft *B. exitiosa* genome has been assembled from next generation sequencing (NGS) of infected flat oyster tissues rather than purified *B. exitiosa* parasites, as sufficient quantities of *Bonamia* could not be generated for purification of the parasite. In addition to the draft *B. exitiosa* genome, the draft genomes of *O. angasi* and a *Poseidonibacter* sp. were generated. This is the first time that drafts of all three genomes have been assembled and provide a potentially valuable resource for further taxonomic comparisons, disease management and selective breeding.

3.2 Introduction

The CSIRO Australian Animal Health Laboratory - Fish Diseases Laboratory (AFDL) collaborative role in this project was to obtain the nucleic acid sequence, compare this with other described *Bonamia* spp. and determine their taxonomic relationship and ensure that available diagnostic tools are suitable.

Prior to 2015, identification of *Bonamia* species in Australia was hampered by the potential existence of both *Bonamia exitiosa* and *Bonamia roughleyi*, with the two being phylogenetically indistinguishable when 18S small-subunit ribosomal RNA (SSU rRNA) DNA sequence was compared. However, Carnegie et al. (2014) determined that *B. roughleyi* was not a *Bonamia* species. In addition, these authors, as well as Hill et al. (2010), concluded that the *Bonamia* sp. detected in South Eastern Australia, by previous investigations (Cochennec-Laureau et al., 2003, Corbeil et al., 2006b) and their own work with *Saccostrea glomerata* and *O. angasi*, was *B. exitiosa*.

The World Organisation for Animal Health (OIE), Manual of Diagnostic Tests for Aquatic Animals has two chapters relating to infection with *Bonamia* species (*B. exitiosa*, Chapter 2.4.2 and *Bonamia ostreae*, Chapter 2.4.3). The *B. exitiosa* chapter describes in detail one conventional PCR developed by Cochennec et al. (2000) that amplifies partial SSU rRNA from both *B. exitiosa* and *B. ostreae* (hereafter referred to in this report as the OIE *Bonamia* PCR). A four base pair deletion and nucleotide mismatches at one locus site within the OIE *Bonamia* PCR amplicon allows distinction between *B. exitiosa* and *B. ostreae* SSU rRNA sequences. Another conventional PCR developed by Carnegie and Cochennec-Laureau (2004) and a real-time TaqMan PCR by Corbeil et al. (2006a) that detects both *B. exitiosa* and *B. ostreae* are also described. The Corbeil et al. (2006a) real-time TaqMan PCR that amplifies partial SSU

rRNA will hereafter be referred to in this report as *Bonamia* sp. qPCR. The *B. ostreae* chapter describes the same three PCRs and includes another real-time TaqMan PCR by Marty et al. (2006) that detects *B. exitiosa* and *B. ostreae*. In addition, the same chapter includes a further conventional PCR by Engelsma et al. (2010) and a SYBR Green real-time PCR by Robert et al. (2009) that detects *B. ostreae*.

The genera *Bonamia* is currently classified within the family Haplosporidiidae and the supergroup Rhizaria. Rhizaria comprises several groups of uncultivable free-living protists with taxonomic relationships that have been inferred almost exclusively from rRNA, actin, and polyubiquitin genes, and remain poorly resolved (Burki et al., 2010). The genomic information publicly available for *B. exitiosa* is limited to rRNA and actin sequences. Valuable new insights into the taxonomic relationship between *Bonamia* spp., Haplospirdiidae and Rhizaria could be gained by the next generation sequencing (NGS) of the *Bonamia* spp. detected in Australian flat oysters.

3.3 Objectives

Objective 1: Obtain nucleic acid sequence and compare with other, described *Bonamia* sp. and determine their taxonomic relationship and ensure that available diagnostic tools are suitable.

3.4 Methods

Ostrea angasi nucleic acid extraction and PCR testing

Ethanol fixed *O. angasi* tissues were received at AFDL and trimmed to obtain a sub-sample of mantle and gill (mg used? Or xmm x xmm piece of tissue?) that was digested overnight in 180 µl ATL buffer and 20 µl Proteinase K (Qiagen) at 56°C. Nucleic acid was extracted from 50 µl of tissue digest using the MagMAX-96™ Viral RNA Isolation Kit on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Life Technologies) with a 50 µl final elution. Real-time PCR assays (Table 1) were performed in a final volume of 25 µl that contained 2 µl extracted nucleic acid, 12.5 µl TaqMan Universal PCR Master Mix and a final concentration of 900 nM for each specific primer, 250 nM for the specific probe. Real-time PCR assays were performed in duplicate on extracted nucleic acids at neat and diluted 10-fold in nuclease free water to overcome the possible presence of PCR inhibitors. The 18S rRNA (Applied Biosystems) real-time PCR was performed to verify the nucleic acid extraction procedure. Real-time PCR assays were performed in a 7500 Fast Real-time PCR system (Life Technologies). Specimens were deemed positive if both duplicates generated a typical amplification curve that crossed the fluorescence threshold of 0.1 during the 45 cycle run. No cycle threshold (C_T) cut-off values were used. Pathogen-specific real-time PCR negative results were considered valid if the control 18S rRNA real-time PCR was positive.

For conventional PCR assays (Table 1), 2 µl extracted nucleic acid template was added to 23 µl reaction mix containing 12.5 µl Hot-StarTaq Master Mix (Qiagen), 360 nM of each primer and molecular grade water. Amplicons for all conventional PCR assays were visualised after electrophoresis on 1.5% agarose gels stained with SYBR Safe DNA Gel Stain (Life Technologies). Amplicons of the expected size were excised from the gel and purified using

the QIAquick Gel Extraction Kit (Qiagen). Each amplicon was sequenced using forward and reverse primers by direct product sequencing using the BigDye Terminator v3.1 Cycle Sequencing chemistry and 3130xl Genetic Analyzer (Applied Biosystems). Chromatogram analysis, consensus sequence generation, BLASTn searches of the NCBI database and multiple sequence alignments were conducted using Geneious (Biomatters).

Table 1. PCR assay primers and probes (where applicable) and cycling conditions described in this chapter

PCR	Primers and Probe (5' – 3')	Cycling Conditions	Source
Bonamia sp. qPCR	ITS-For CCCTGCCCTTTGTACACACC ITS-Rev TCACAAAGCTTCTAAGAACGCG Bon-ITS 6FAM-TTAGGTGGATAAGAGCCGC- MGB	1x 50°C for 2min 1x 95°C for 10min 45x 95°C for 15sec 63.6°C for 1min	Corbeil et al., 2006a
18S rRNA qPCR	18S Forward CGGCTACCACATCCAAGGAA 18S Reverse GCTGGAATTACCGCGGCT 18S Probe VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA	1x 50°C for 2min 1x 95°C for 10min 45x 95°C for 15sec 60°C for 1min	Applied Biosystems Cat# 4308329
OIE *Bonamia* sp. PCR	Bo CATTTAATTGGTCGGGCCGC Boas CTGATCGTCTTCGATCCCCC	1x 95°C for 15min 40x 94°C for 1min 55°C for 1min 72°C for 1min 1x 72°C for 7min	Cochennec et al., 2000
B. ostreae-specific PCR	BOSTRE-F TTACGTCCCTGCCCTTTGTA BOSTRE-R TCGCGGTTGAATTTTATCGT	1x 95°C for 15min 35x 94°C for 30sec 55°C for 30sec 72°C for 30 sec 1x 72°C for 7min	Ramilo et al., 2013
B. exitiosa specific PCR	BEXIT-F GCGCGTTCTTAGAAGCTTTG BEXIT-R AAGATTGATGTCGGCATGTCT	1x 95°C for 15min 35x 94°C for 30sec 58°C for 30sec 72°C for 30sec 1x 72°C for 7min	Ramilo et al., 2013
Haplosporidia sp. PCR	haploITSf GGGATAGATGATTGCAATTRTTC ITS-B TATGCTTAAATTCAGCGGGT	1x 95°C for 15min 40x 94°C for 30sec 50°C for 30sec 72°C for 1.5min 1x 72°C for 7min	Carnegie et al., 2014
Perkinsus sp. qPCR	T-PERK-f TCCGTGAACCAGTAGAAATCTCAAC T-PERK-r GGAAGAAGAGCGACACTGATATGTA T-PERK-probe 6FAM-GCATACTGCACAAAGGG-MGB	1x 50°C for 2min 1x 95°C for 10min 45x 95°C for 15sec 63.6°C for 1min	Gauthier et al., 2006

Next Generation Sequencing

In preparation for NGS, nucleic acid was extracted from ethanol fixed or fresh *O. angasi* mantle and gill tissue following overnight digestion in ATL and Proteinase K with the QIAamp DNA Mini Kit (Qiagen). Extracted nucleic acids were quantified by Qubit dsDNA HS Assay Kit (Invitrogen) before packaging and shipment to Macrogen Inc. (South Korea) for NGS. The quantity and quality of the samples were verified upon arrival and after the preparation of cDNA libraries with the Truseq nano DNA kit (350 bp). After passing cDNA library quality

parameters, the samples were sequenced with the Hiseq X Ten 150 bp paired end platform (Illumina). DNA raw data sequences were received on hard disk drives that, upon arrival, were loaded onto the CSIRO research cloud in backed-up storage, prior to analysis.

Ostrea angasi reference genome assembly and annotation

B. exitiosa was unable to be sufficiently purified for direct sequencing of the parasite. Therefore, the only way to obtain genomic information from the parasite was by first obtaining a 'clean' reference *O. angasi genome* and comparing this to genomic information obtained from an *O. angasi* sample infected with *Bonamia*. An *O. angasi* sample (18-01525-0003) not infected with *Bonamia* was selected for NGS to obtain a 'clean' reference oyster genome. Subsequently NGS was performed on *B. exitiosa* infected *O. angasi* tissue (16-00979-0005 and 16-00730-0003; samples that contained a mix of oyster and parasite DNA). These data were used to differentiate oyster DNA from parasite DNA.

The raw DNA sequencing reads from the HiSeq X Ten sequencing platform from an uninfected *O. angasi* oyster were cleaned by removing Illumina adapters, removing sequences with a quality score lower than 20, and discarding sequences with a final length shorter than 50 bp using Trimmomatic v.0.36 (Bolger et al*.,* 2014) . K-mer profiles were constructed from the cleaned reads using BBmap v.38.00 (https://jgi.doe.gov/data-and-tools/bbtools/) and a k-mer length of 31. The k-mer profiles were used to estimate genome size, heterozygosity and repeat content with GenomeScope (Vurture et al., 2017). Due to a moderately high estimate of genome heterozygosity (0.6%), the cleaned reads were assembled using Platanus v.1.2.4 (Kajitani et al., 2014), which is designed to handle high-throughput data of heterozygous samples. The genome was assembled using three Platanus modules, *assemble, scaffold* and *gap_close,* with the default parameters (Kajitani et al., 2014).

The assembled *O. angasi* genome was annotated using the MAKER pipeline v.2.31.8 (Cantarel et al., 2008). First, repeats were annotated and masked with RepeatMasker v.4.0.6 (www.repeatmasker.org). The masked genome was subsequently annotated using homology-based gene prediction, combined with *ab initio* methods within MAKER (Cantarel et al., 2008). Homology-based gene prediction was used with EST and protein sequences from *Crassostrea gigas* (Pacific oyster genome GCF_000297895.1) and *Crassostrea virginica* (Eastern oyster genome GCF_002022765.2). The *ab initio* gene predictors GeneMark.hmm v.3.47 (Besemer, 2001) and SNAP v.2.31.8 (Korf, 2004) were used with Hidden Markov Models (HMM) trained using *Caenorhabditis elegans* and *Drosophila melanogaster* gene sets. AUGUSTUS v.3.2.3 (Stanke and Morgenstern, 2005) HMM were trained using *C. elegans*. The homology-based and *ab initio* predictions were then combined into a non-overlapping set of gene models in MAKER (Cantarel et al., 2008). The completeness and contamination of the final genome was estimated using the Eukaryota 'odb9' essential single-copy orthologs with BUSCO v.3.0.2 (Simão et al., 2015).

Bonamia exitiosa genome assembly and annotation

A process of 'differential coverage binning' was utilised to obtain the *B. exitiosa* genome sequence from the mixed metagenome samples 16-00979-0005 and 16-00730-0003 (Albertsen et al., 2013). This procedure relied on the fact that all DNA sequences originating from the same organism should have a similar abundance, and this abundance should follow

a normal distribution. For example, if *B. exitiosa* cells were more abundant in a particular sample than *O. angasi* cells, then DNA sequences originating from the *B. exitiosa* genome should also be consistently more abundant. That holds true for other organisms that may unintentionally be present in the sample and allows the sample to be 'cleaned' of unwanted contaminating sequences.

The raw DNA sequencing reads from the HiSeq X Ten sequencing platform for the two *O. angasi* oysters infected with *B. exitiosa* (16-00730-0003 and 16-00979-0005) were cleaned by removing Illumina adapters, trimming sequence with a quality score lower than 20, and discarding sequences with a final length shorter than 50 bp with Trimmomatic v.0.36 (Bolger et al., 2014). For the differential coverage binning procedure, the two samples need to be combined into a single master assembly of consensus contigs. To reduce redundancy in the combined dataset, the reads were first normalised to a target of 25x using BBnorm v.38.00 (https://jgi.doe.gov/data-and-tools/bbtools/). The normalised data set was then assembled with a k-mer size of 64 in ABySS v.2.0.2 (Jackman et al., 2017). To generate coverage profiles, sequencing reads from the two samples were individually mapped to the master assembly using Bowtie v.2.2.9 (Langmead & Salzberg, 2012). Normalised coverage values (RPKM; Reads per Kilobase Million) were calculated from the mapping files using *pileup.sh* within BBnorm v.38.00 (https://jgi.doe.gov/data-and-tools/bbtools/). The GC content and length of each contig was obtained using custom Python v.3.5.2 scripts. To identify clusters of contigs that originated from the *B. exitiosa* genome, the *B. exitiosa* small subunit rRNA (EU016528.1) and actin (KM073107.1) genes were detected in the master assembly using BLASTn v.2.2.31 (Altschul et al., 1990). The data was visualised by plotting the coverage profiles of the two samples against each other, which was then overlaid with GC content, length and marker genes using ggplot v3.0.0 (Wickham, 2009) in the R programming environment v.3.4.4.

After removal of contigs matching to the *O. angasi* reference genome, two probable genome clusters were apparent. One of the clusters had a low GC content (~ 25%) and contained all of the *B. exitiosa* marker genes (SSU rRNA and actin), and therefore was identified as containing sequences highly-likely to represent the *B. exitiosa* genome. This cluster was extracted from the master assembly for further cleaning and annotation. First, the uninfected oyster reads were mapped against the putative *B. exitiosa* contigs using Bowtie v.2.2.9 (Langmead & Salzberg, 2012) and any contigs that were mapped were removed. Although the clean *O. angasi* genome had already been used to remove oyster contigs, the process of using unassembled reads (rather than final contigs, which may not contain all reads) provided an extra level of stringency ensuring that oyster contigs were not present in the *B. exitiosa* contigs.

The final *B. exitiosa* contigs were annotated using the MAKER pipeline v.2.31.8 (Cantarel et al., 2008) in the same manner as for the *O. angasi* genome. The only difference was the reference organisms used for the homology-based predictions and for training the HMM. In this case, *Plasmodiophora brassicae* (genome GCA_002093825.1) and *Plasmodium falciparum* (genome GCF_000002765.4) were chosen for protein homology annotations (based on small-scale tests to determine closely related organisms). *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* HMM were used for GeneMark.hmm v.3.47 (Besemer 2001), *A. thaliana* and *Oryza sativa* HMM were used in SNAP v.2.31.8 (Korf, 2004), and a *P. falciparum* HMM was used for AUGUSTUS v.3.2.3 (Stanke and Morgenstern 2005). Due to the lack of genomes closely related to *B. exitiosa*, all non-overlapping *ab initio* gene models were

retained, regardless of whether they had EST or protein homology evidence. The predicted *B. exitiosa* genes were compared to the NCBI non-redundant (nr) database (accessed 11.09.2018) using BLASTp v.2.2.31 (Altschul et al., 1990). Best matches to the *B. exitiosa* genes were extracted and examined in a phylogenetic context (based on the topology of NCBI taxonomy) using ETE3 v.3.0.0 (Huerta-Cepas et al., 2016) in Python v.3.5.2. The genes were annotated with Gene Ontology terms (Ashburner et al., 2000) using InterProScan v.5.24 (Jones et al., 2014).

Poseidonibacter sp. genome assembly and annotation

The 'differential coverage binning' process unexpectedly revealed that another genome was present in both of the *B. exitiosa*-infected oyster samples. As for *B. exitiosa*, these contigs were extracted from the master assembly for identification and annotation. In this case, a preliminary analysis revealed that the genome was likely bacterial in origin. Thus, Prodigal v.2.6.3 (Hyatt et al., 2010), which is optimised for prokaryotic genomes, was used to predict genes using default settings. The predicted bacterial genes were compared to the NCBI nr database (accessed 11.09.2018) using BLASTp v.2.2.31 (Altschul et al., 1990). The completeness and contamination of the final genome was estimated using the 'Delta + Epsilon proteobacteria' odb9 essential single-copy orthologs with BUSCO v.3.0.2 (Simão et al., 2015).

3.5 Results

Confirmation of *Bonamia exitiosa* in *Ostrea angasi* from Victoria, New South Wales and South Australia

As part of the annual surveillance of cultured *O. angasi* in Victoria in 2013 and 2014, 24 of 105 animals tested were positive by the *Bonamia* sp. qPCR with C_T values ranging from 25.5 to 37.9. Two positive samples from 2013 and three from 2014 were also tested with the OIE *Bonamia* PCR and amplicons were Sanger sequenced and analysed. Positive detections from both submissions were identical in DNA sequence and reported as *Bonamia* sp. However, based on the information later published by Carnegie et al. (2014) and subsequent re-analysis of the SSU rRNA sequences amplified by the OIE *Bonamia* PCR, *B. exitiosa* had been detected. In 2015, 21 of the 87 samples in four submissions for the first surveillance of *O. angasi* from Victoria that year were positive with C_T values ranging from 28.0 to 40.2 when tested by the *Bonamia* sp. qPCR. Of these, four samples were tested by the OIE *Bonamia* PCR and the resulting amplicons Sanger sequenced. All four DNA sequences were 100% identical to each other and several *B. exitiosa* reference sequences i.e. AF337563 (New Zealand) and DQ312295 *Bonamia* sp. (New South Wales). A further 5 submissions of surveillance samples from Victoria were submitted in 2015 with 49 of 106 samples positive by *Bonamia* sp. qPCR with C_T values ranging from 23.1 to 40.2. Twelve of these samples were positive when tested by the OIE *Bonamia* PCR. Sanger sequencing of the amplicons again found the sequences were 100% identical to each other and several *B. exitiosa* reference sequences. Upon reporting the *Bonamia* sp. detected in 2015 as *B. exitiosa* an Aquatic Consultative Committee for Emerging Animal Disease (AqCCEAD) was convened.

As part of the AqCCEAD investigation to confirm the identification of *B. exitiosa* detection in *O. angasi* in Victorian farms, several additional conventional PCRs were performed, including

B. ostreae-specific and *B. exitiosa*-specific conventional PCRs, that amplify partial SSU rRNA and partial ITS1 (Ramilo et al., 2013). Both tests were performed on 94 *Bonamia* sp. qPCR positive samples (ranging in C_T value from 18.7 to 41) between 2013 and 2015. All samples were negative by the *B. ostreae*-specific PCR. When tested with the *B. exitiosa*-specific PCR all 56 samples with C_T values <34 were positive as were a further 8 of the remaining 38 samples with C_T values >34. Of these, 18 amplicons were Sanger sequenced and had 98.1 to 100 % identity with each other and several *B. exitiosa* sequences i.e. EU709074 and AF262995 (New Zealand) and D1312295 *Bonamia* sp. (New South Wales). The Carnegie et al. (2014) *Haplosporidia* sp. PCR was also performed as it amplified a region from the 18S small-subunit (SSU) rRNA to the 18S large-subunit (LSU) rRNA that included the highly variable sequences in ITS1 and ITS2 regions. Of the 15 amplicons Sanger sequenced the nucleotide identity ranged from 98.1 to 100% with each other and *B. exitiosa* reference sequences such as that from New Zealand (EU709074). The information gathered resulted in a 2016 OIE notification of *B. exitiosa* confirmed detection within Australia.

In December 2015, *O. angasi* ethanol fixed samples were submitted from 3 farms in South Australia for *Bonamia* sp. surveillance. The 120 samples originated from three farms including one that historically had high mortalities. Extracted nucleic acid from each sample was tested with the *Bonamia* sp. qPCR. On the first farm 16 samples were test positive with C_T values ranging from 28.1 to 42.7. The 16 *Bonamia* sp. qPCR positives were tested by the OIE *Bonamia* PCR and 10 generated amplicons of the expected size. Three amplicons were Sanger sequenced and the 264 bp primer trimmed DNA sequences analysed. All three sequences were 100% identical to *B. exitiosa* reference sequences i.e. AF337563 (New Zealand), DQ312295 *Bonamia* sp. (New South Wales) and *B. exitiosa* from Victoria (2013 to 2015 submissions). Of the 25 samples tested from the second farm, 4 were positive with C_T values ranging from 38.4 to 42.2. Of the four *Bonamia* sp. qPCR positives, one was positive by the OIE *Bonamia* PCR, however, insufficient amplicon was amplified to generate a Sanger sequence. From the samples tested from the third farm, 27 were positive with C_T values ranging from 32.5 to 44.4. Of the 27 *Bonamia* sp. qPCR positives, 16 were positive by the OIE *Bonamia* PCR. Three amplicons were Sanger sequenced and the DNA sequences generated were 100% identical with *B. exitiosa* reference sequences.

In 2017, 32 gill samples fixed in ethanol from healthy *O. angasi* originating from a farm in NSW were tested. Four of the samples were positive by the *Bonamia* sp. qPCR with C_T values ranging from 34.3 to 38.2. Two of the positive samples were tested by the OIE *Bonamia* PCR and 1 generated an amplicon that was Sanger sequenced and the 264 bp primer trimmed DNA sequence analysed. The sequence was 100% identical to *B. exitiosa* reference sequences i.e. AF337563 (New Zealand), DQ312295 *Bonamia* sp. (New South Wales) and *B. exitiosa* from Victoria (2013 to 2015 submissions) and *B. exitiosa* South Australia (2015 submission).

Whole Genome Sequencing of *Bonamia exitiosa*

Selection of samples for Next Generation Sequencing

The samples to be utilised for genome sequencing of *B. exitiosa* were originally proposed to consist of the parasite purified from fresh *O. angasi*. However, several attempts to purify the *B. exitiosa* at AFDL and SARDI provided insufficient *B. exitiosa* parasite for nucleic acid extractions and NGS. This was most likely due to the uncertain infection status of the oysters

and the limited amounts of tissue available for purification. Therefore, an alternate approach was formulated that involved identifying *O. angasi* samples with the lowest C_T values when screened by the *Bonamia* sp. qPCR. New extractions of DNA would be prepared and submitted for NGS by the HiSeq X Ten platform. This meant that only *B. exitiosa* detected in Victoria could be used as the positive samples from South Australia and New South Wales had much higher C_T values and therefore much less *B. exitiosa* would be present in those samples.

Of the hundreds of Victorian samples submitted to AFDL for testing from Victorian farms (2011 to 2016) and the tank trail from this project, two tank trial samples (16-00979-0005 and 16-00730-0003) submitted in 2016 were identified to have the lowest detected *Bonamia* sp. qPCR mean C_T values of 14.02 and 14.98, respectively. Both samples were from mortalities observed during the Queenscliff laboratory tank trial and both the oysters originated from Victoria. Ethanol fixed tissues of mantle and gill had been submitted to AFDL for both animals. New extractions of DNA from preserved mantle tissue were performed for both samples and when tested by the *Bonamia* sp. qPCR, returned C_T values of 15.27 and 15.15 respectively.

Prior to NGS, the presence of *B. exitiosa* in both samples was confirmed by performing the *Haplosporidia* sp. PCR and subsequent Sanger DNA sequencing and analysis. Due to the presence of some ambiguous nucleotides in the sequence obtained, 16-00979-0005 and 16-00730-0003 shared 98.5% identity. Both sequences shared >97.3% identity with *B. exitiosa* New Zealand reference sequences (JF831656 and EU709074). When MegaBLAST was performed against the GenBank database, the top hit with 97% identity and 99% coverage, was *B. exitiosa* (JF831664 New Zealand). Both 16-00730-0003 and 16-00979-0005 sequences shared 68% or less identity with *B. ostreae* and *Bonamia perspora* reference sequences (EU709108 and EU709119 USA). The DNA was negative for *Perkinsus* sp. when tested by the *Perkinsus* sp. qPCR (Gauthier et al. 2006) that detects all *Perkinsus* species. This assay was conducted to ensure that *Perkinsus* (another protozoan) was not present in the DNA. The presence of another protozoan would have made identification of *Bonamia* specific DNA from the NGS data more complicated.

The disadvantage of this sequencing approach was the high number of reads from *O. angasi* as well as other organisms that may have been present in samples 16-00730-0003 and 16-00979-0005, other than *Bonamia*. Therefore, HiSeq X Ten data was generated from an *O. angasi* sample that was negative for *B. exitiosa* and this data would be assembled into multiple contigs that could be read-mapped from the data already generated for samples positive for *B. exitiosa*. Only the unmapped reads would be retained and the remaining dataset therefore significantly enriched with *B. exitiosa* sequences. To facilitate this strategy, nucleic acid was extracted from an ethanol fixed *O. angasi* gill sample (15-01238-0017) where that sample and all the others collected from the Victorian farm in 2015 were *Bonamia* sp. qPCR negative. The new nucleic acid extraction from the sample was again negative when tested by the *Bonamia* sp. qPCR but was importantly positive by the extraction control qPCR. However, upon submission of the nucleic acid to the commercial company sourced for HiSeq X Ten sequencing, the DNA was found to be too degraded to perform the sequencing run. Therefore additional *O. angasi* were sourced fresh from a farm in Victoria and nucleic acid extracted from individual animals without ethanol fixation. One nucleic acid sample was

selected (18-01525-0003) that was negative by the *Bonamia* sp. qPCR and positive by the extraction control qPCR. The nucleic acid from 18-01525-0003 was successfully sequenced with the HiSeq X Ten platform.

Next Generation Sequencing and Assembly

More than 1 billion total DNA sequences were obtained from infected and uninfected *O. angasi* tissue by NGS with the HiSeq X Ten platform in an attempt to obtain a draft *B. exitiosa* genome (Table 2). A stringent quality trimming procedure filtered 6 – 19% of the sequences due to low quality, resulting in more than 150 million high-quality sequence pairs for each sample (Table 2).

A differential coverage binning procedure was used to detect and assemble the genomes of organisms present in the infected oyster samples (**Figure 1**). In addition to the oyster host and *Bonamia* parasite, this procedure also revealed the presence of a bacterial species, which was identified as belonging to the genus *Poseidonibacter* (Epsilonproteobacteria; Campylobacteraceae) (**Figure 1**).

Table 2. Sample type and sequencing read quality

SAN #	Status	Raw read pairs	Cleaned pairs
16-00730-0003	*B. exitiosa* infected *O. angasi*	198,356,079	160,355,338 (81%)
16-00979-0005	*B. exitiosa* infected *O. angasi*	187,299,887	156,949,378 (84%)
18-01525-0003	Clean *O. angasi* (without *B. exitiosa*)	250,688,423	235,336,595 (94%)

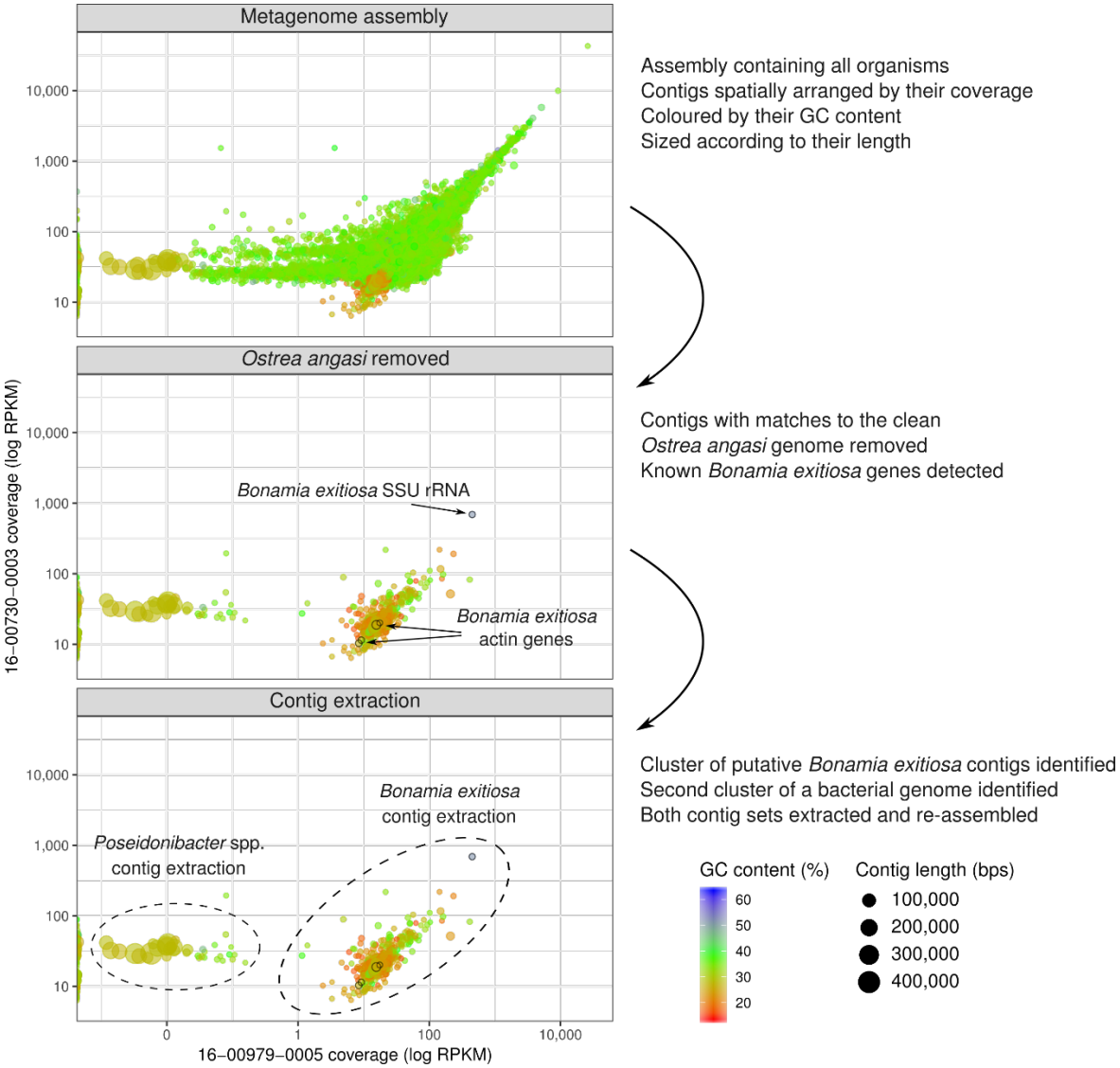

Figure 1. Differential coverage binning procedure used to obtain the partial *Bonamia exitiosa* genome and an unexpected bacterial genome.

Ostrea angasi reference genome assembly and annotation

An uninfected *O. angasi* sample (18-01525-0003) was sequenced and its genome assembled to help subtract host DNA from *Bonamia*-infected samples (16-00730-0003 and 16-00979-0005) in the binning procedure (Figure 1, Table 2 and Table **3**). The final putative O. angasi genome was 672 Mb in size (Table 3), which is similar to the size of 717 Mb (177 Mb in repeats) estimated by k-mer profiling, and is also similar to other sequenced oyster genomes, including *C. virginica* (684 Mb) and *C. gigas* (558 Mb). The number of predicted protein coding genes in *O. angasi* (72,073) is higher than *C. virginica* (60,213) and *C. gigas* (46,753), although this is likely an overestimate in the prediction models and would be more accurate with additional data (i.e. RNA-Seq). Using a set of essential single-copy eukaryotic genes, the genome was estimated to be ~ 85% complete with very low levels of contamination (~ 0.3%) (Table 3).

Bonamia exitiosa genome assembly and annotation

The *B. exitiosa* genome was identified during the differential coverage procedure based on its abundance, GC content, and the presence of marker genes (Figure 1). The final genome size was 9.5 Mb, had a low GC content (25.7%), and contained a predicted 8,007 protein coding genes (Table 1). Unfortunately, it is difficult to estimate the completeness of the *B. exitiosa* genome or to evaluate these numbers in context due to the absence of closely related genome sequences. Indeed, this is not only the first *Bonamia* genome to be generated, it is also the first genome to be generated from the Haplosporidia clade. Nonetheless, genomes with the highest level of relatedness were attempted to be identified by comparing the *B. exitiosa* genes to genomes available on NCBI (Table 4; Figure 2).

This analysis showed that *Plasmodiophora brassicae*, which causes the plant disease 'clubroot', had the most closely related genes to *B. exitiosa* (Table 4). *Plasmodiophora brassicae* is indeed classified in the same supergroup of eukaryotes as *Bonamia* (Rhizaria) and the same phylum (Cercozoa), although it belongs to a different class (Phytomyxea). The *P. brassicae* genome is 24.4 Mb and contains 9,727 protein coding genes (Schwelm et al., 2015), which is larger than the *B. exitiosa* genome but contains an approximately similar number of genes (Table 4). In contrast to *B. exitiosa*, the *P. brassicae* genome has a higher GC content of 59.1% (Schwelm et al., 2015). Another species with a number of closely related genes was *Reticulomyxa filosa*, which is also part of the Rhizaria but does not belong to the Cercozoa. The *R. filosa* genome is 101.9 Mb and contains an estimated 39,963 genes (Glöckner et al., 2014), which is far larger than either *B. exitiosa* or *P. brassicae* (Table 4) and underscores the problem of estimating genome completeness using only distantly related species. Nevertheless, this data supports the current inclusion of *Bonamia* within the Rhizaria and Cercozoa clades. Other species with a high number of gene matches to *B. exitiosa* included *Aphanomyces astaci*, which is an aquatic water mould that causes the disease crayfish plague (Makkonen et al., 2016) (Table 4). More broadly, the *B. exitiosa* genes were taxonomically related to a diverse range of organisms, including plants (Viridiplantae), amoeba (Amoebozoa), fungi, Oomycetes (water moulds), algae (Geminigeraceae and Cyanidiaceae), and *Plasmodium* parasites (Plasmodiidae) (Figure 2).

Of the 8,007 *B. exitiosa* genes, 3,540 (44.2%) could be annotated as a known protein. Functionally, the genes were involved in molecule transport, proteolysis and oxidation-reduction processes, which may be a reflection of its parasitic intracellular environment (Figure 3). Indeed, ensuring oxidation-reduction equilibrium is critically important for parasite pathogenicity. For example, the malaria parasite, *P. falciparum*, uses two major oxidation-reduction enzymes to survive the host environment, and these have been suggested as potential targets for new therapeutic strategies (Kehr et al., 2010).

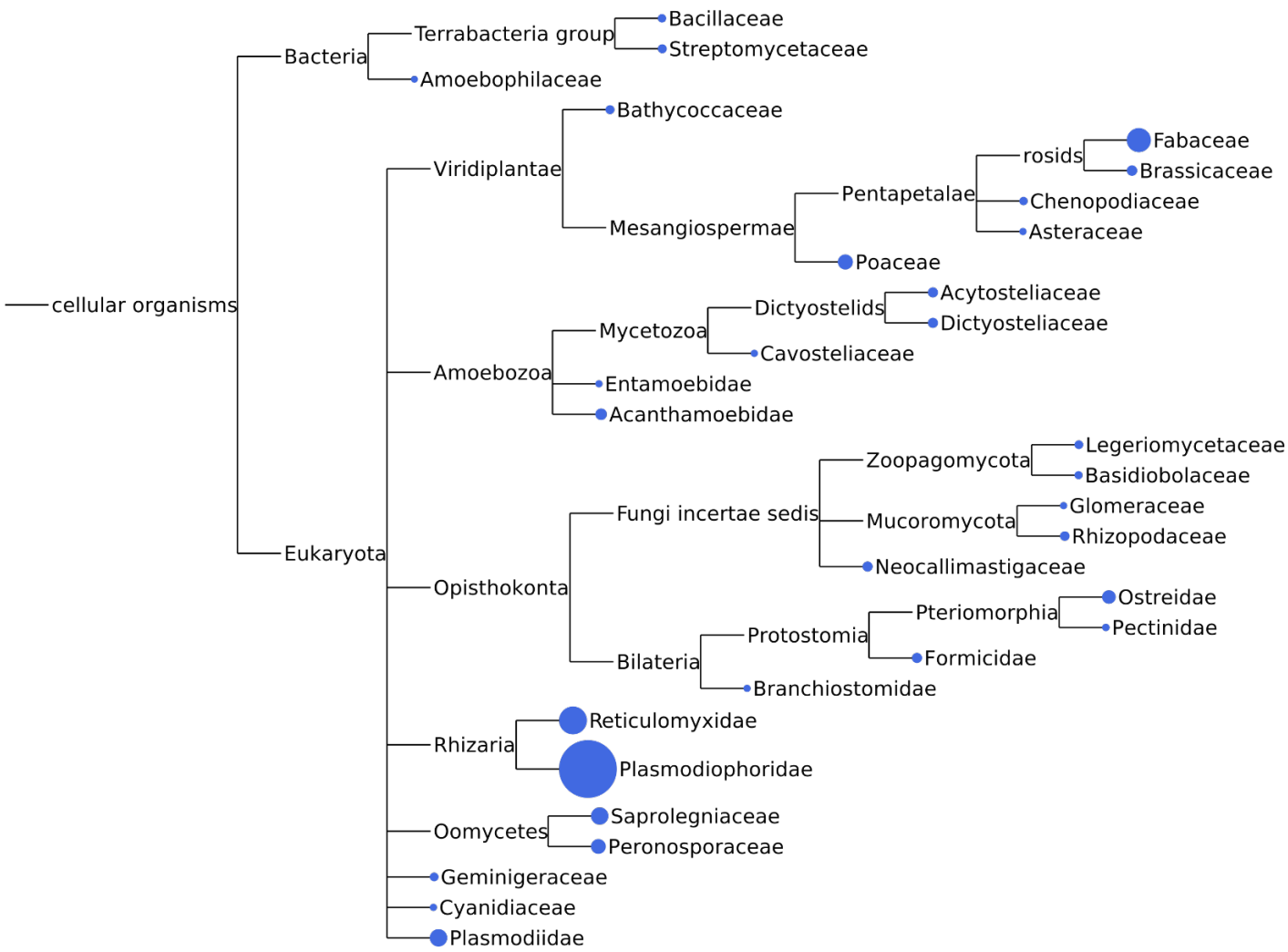

Figure 2. Phylogenetic topology of the best family-level matches to the predicted Bonamia exitiosa genes (the size of the blue circles are proportional to the number of hits to that particular family and the relationships between families was inferred from the NCBI taxonomic database).

Table 3. Genome sequencing, assembly and annotation of *Ostrea angasi*, *Bonamia exitiosa* and *Poseidonibacter* sp.

	Assembly size (Mb)	Scaffolds	Scaffold N50 (bps)	Max scaffold (bps)	GC content (%)	Estimated completeness (%)	Predicted genes
Ostrea angasi	672.2	95,628	11,926	142,686	35.2	84.2	72,073
Bonamia exitiosa	9.5	1,550	9,470	51,155	25.7	ND	8,007
Poseidonibacter sp.	3.5	30	212,045	421,818	28.1	95.0	3,449

ND - not determined due to a lack of genome sequences from closely related organisms

Table 4. Organisms in the NCBI database with genes most similar to the *Bonamia exitiosa* genes.

Genes with highest similarity	Total gene homologues	Species	Common name	Genome size (Mb)	Number of genes	Basal classification
194	2,577	*Plasmodiophora brassicae*	Clubroot	24.4	9,727	Rhizaria
91	2,215	*Reticulomyxa filosa*	N/A	101.9	39,963	Rhizaria
35	2,332	*Acanthamoeba castellanii*	N/A	46.7	14,974	Amoebozoa
32	2,455	*Vigna angularis*	Adzuki bean	444.4	37,769	Angiosperms
26	2,467	*Crassostrea virginica*	Eastern oyster	684.7	60,213	Mollusca
25	2,314	*Guillardia theta*	N/A	87.1	24,822	Cryptophyta
23	2,444	*Basidiobolus meristosporus*	N/A	96.7	16,110	Fungi
22	2,438	*Aphanomyces astaci*	Crayfish plague	51.8	26,259	Heterokonta
21	2,504	*Mizuhopecten yessoensis*	Japanese scallop	987.6	41,567	Mollusca
20	2,464	*Planoprotostelium fungivorum*	N/A	38.2	17,124	Amoebozoa

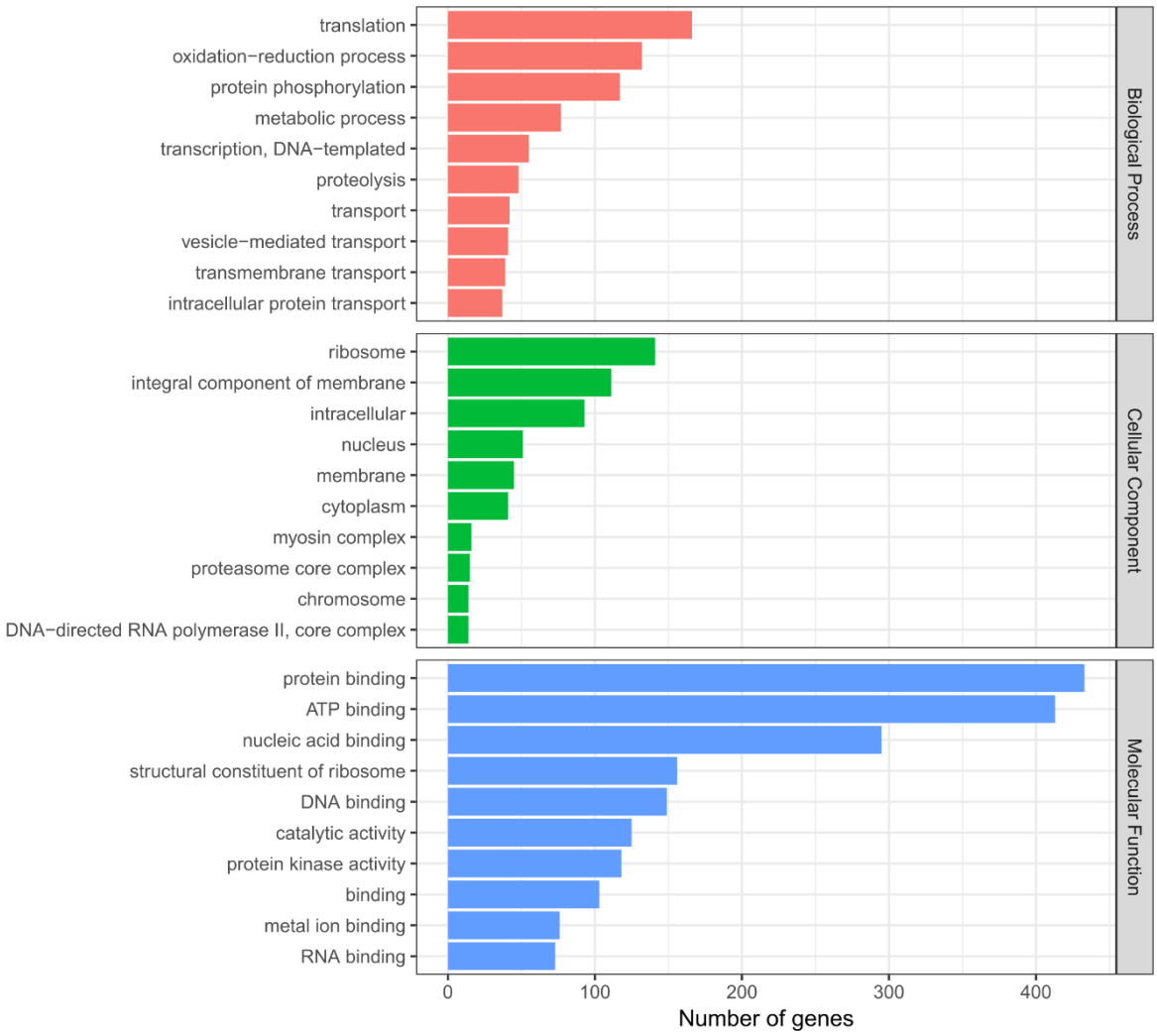

Figure 3: Most common high-level functions of the *Bonamia exitiosa* genes (functions were determined based on the Gene Ontology hierarchy (Ashburner et al., 2000) which contains three sub-classifications, Biological Process, Cellular Component and Molecular Function).

Poseidonibacter *sp. genome assembly and annotation*

Unexpectedly, a bacterial genome was also detected in the *B. exitiosa*-infected oyster samples, which was subsequently identified as a *Poseidonibacter* species. The genome was 3.5 Mb, contained 3,449 genes and was estimated to be 95% complete (Table 3). Moreover, the genome was assembled into only 30 scaffolds and had a large scaffold N50, suggesting a high-quality genome assembly (Table 3). Additional genome-wide analysis showed that 85% of the genes (2,896 of 3,395) were most closely related to genes in the *Poseidonibacter lekithochrous* genome (formally *Arcobacter lekithochrous*) (Pérez-Cataluña et al., 2018; Diéguez et al., 2017), indicating a very close relationship. Interestingly, *P. lekithochrous* was isolated from the great scallop (*Pecten maximus*) in a Norwegian hatchery during an experiment to study the microbiota of the scallop throughout its reproductive cycle (Diéguez et al., 2017). The *Poseidonibacter* sp. detected here was present in both *B. exitiosa*-infected oyster samples but was not detected in the clean oyster sample. It is possible that this is an

opportunistic bacterium that proliferates in oysters already weakened by the *Bonamia* infection, although there is no suggestion from the literature that *Poseidonibacter* is pathogenic (Diéguez et al., 2017). Additional experiments may elucidate the role of this bacterium, if any, in the infection of *O. angasi* with *B. exitiosa*.

3.6 Discussion

The current diagnostic PCR testing strategy for the detection and confirmation of *Bonamia* species in *O. angasi* samples at AFDL involves initially screening the samples with the *Bonamia* sp. qPCR. Positive samples with the lowest C_T values are preferentially tested with OIE *Bonamia* PCR to increase the likelihood of generating amplicons sufficient for Sanger DNA sequencing and analysis, to confirm the qPCR results. As a result, the *Bonamia* sp. detected in Victoria between 2013 and 2015, South Australian in 2015 and New South Wales in 2017 were all confirmed to be *B. exitiosa*. In addition, further PCR tests such as *B. ostreae*-specific, *B. exitiosa*-specific and *Haplosporidia* sp. PCRs have been demonstrated to provide additional confirmation of the *Bonamia* sp. detected. Therefore, the current diagnostic tools have been demonstrated to be sufficient and effective in the detection and identification of *Bonamia* species from farmed *O. angasi* in southern Australian waters as *B. exitiosa*.

A draft genome for *B. exitiosa* was successfully assembled for the first time from the NGS data generated during this project. The *B. exitiosa* genome was assembled to further characterise the taxonomic relationship of the organism. The 9.5 Mb draft genome assembly represents the first available genomic information from the entire Haplosporidia lineage. Therefore, further evaluation of the relationship between *B. exitiosa*, *B. ostreae* and *B. perspora*, all known to infect oysters, was unable to be investigated further due to a lack of genomic sequence for the other *Bonamia* species. However, the 8,007 *B. exitiosa* genes that were identified are a significant contribution toward understanding this parasite and the disease it causes, particularly considering that only two *B. exitiosa* genes had previously been identified (i.e. SSU rRNA and actin). These genes can now be mined to gain a greater understanding of the pathogenesis mechanisms of *B. exitiosa* and used in a comparative genomics context with other single-celled parasites. For instance, the predicted *B. exitiosa* genes were most related to other members of the Rhizaria, as well as single-celled algae, amoeba, fungi and plants, which is consistent with the lifecycle of *Bonamia* parasites. Functionally, the genes tended to be involved in molecule transport and binding, proteolysis and oxidation-reduction processes, which are likely to be important in the parasite's intracellular environment. The completeness of the draft *B. exitiosa* genome and its 8007 protein coding genes was difficult to fully evaluate in context, due to the absence of closely related genomic sequences. To obtain a higher level of confidence in the assembled genome and its genes, additional sequencing approaches would be required such as sequencing messenger RNA from *B. exitiosa* infected *O. angasi*. Further work to generate *O. angasi* that are heavily-infected with *B. exitiosa* should be considered to enable *B. exitiosa* to be purified for whole genome sequencing. All

sequences generated would then be specific for the parasite, which would enable refinement and completion of the whole-genome sequence.

To aid in the differentiation of *B. exitiosa* from the infected *O. angasi* host, an uninfected *O. angasi* was sequenced. Fortuitously the sequences assembled into a largely complete genome draft for *O. angasi* with a similar number of genes to other oysters that have genomes available. Despite the 72,073 genes likely to be an overestimation from the prediction models that would benefit from additional data such as messenger RNA sequencing, it should still be a good draft for use in future projects. For instance, the *O. angasi* draft genome and predicted gene models may be useful in selective breeding programs or for use in comparative genomics studies with other commercially important oyster species.

The identification of a nearly complete *Poseidonibacter* genome in the two *B. exitiosa* infected *O. angasi* samples sequenced was unexpected. This bacterium could simply by an adventitious detection of an organism present in the tank trial from which the samples were collected or possibly an opportunistic bacterium that proliferates in oysters already weakened by the *Bonamia* infection. However, there is no suggestion from the literature that *Poseidonibacter,* to date, is pathogenic (Diéguez et al., 2017). Additional experiments may elucidate the role of this bacterium in the infection of *O. angasi* with *B. exitiosa*. Initially, a simple correlation between the presence of *B. exitiosa* and the presence of *Poseidonibacter* in flat oysters could be conducted, which would quickly determine if the relationship is consistently observed.

3.7 Conclusion

This project has been successful in accomplishing its planned objectives:

- The *Bonamia* sp. present in *O. angasi* in southern Australian waters including Victoria, South Australia and New South Wales was successfully confirmed by PCR and DNA sequencing to be *B. exitiosa*. This analysis verifies that the current diagnostic tools are suitable for identification of *Bonamia* species.

- For the first time, a draft genome was assembled for a member of the family Haplosporidiidae, from NGS data for *B. exitiosa.* The genome was estimated to be 9.5 Mb and encode 8007 genes. Analysis of the genes phylogenetically placed *B. exitiosa* as expected within the Rhizaria. The *B. exitiosa* genome will aid comparison with other *Bonamia* species as their genomes become available.

- For the first time, a draft genome was assembled from NGS data for the Australian flat oyster *O. angasi*. The genome was estimated to be 672 Mb with a predicted 72,073 genes. The *O. angasi* genome will aid future breeding programs and comparative genomics studies.

- Unexpectedly, a nearly complete genome was assembled for an Epsilonproteobacterium, *Poseidonibacter* from NGS data generated from *O. angasi* tissues infected with *B. exitiosa*.

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4. Agriculture Victoria

Determining risk factors for clinical bonamiosis and validation of the *Bonamia* sp. qPCR.

4.1 Introduction

The project objective for Agriculture Victoria was to:

- Improve understanding of bonamiosis infestations in Native Oysters including the determination, under controlled conditions, of the stressors that induce clinical disease in subclinically infected oysters.

Through the process of conducting this project ancillary work that was undertaken included:

- Determining the diagnostic sensitivity and specificity of histopathological examination and a real-time polymerase chain reaction (qPCR) test for the diagnosis of *Bonamia* sp. infection in farmed Native Oysters;
- Calculating the optimal qPCR cycle threshold (C_T) epidemiological cut-point for classification of positive and negative cases of *Bonamia* sp. in farmed Native Oysters; and
- Undertake initial surveillance of cultured *O. angasi* stock in Tasmania and NSW

4.2 Methods

The project involved four individual trials; two field trials undertaken at the Grassy Point Aquaculture Reserve (2016 and 2017), which had a history of significant oyster deaths presumably due to *Bonamia* sp. infection, and two tank trials undertaken at the Marine and Freshwater Discovery Centre (Queenscliff in 2016 and 2017).

Tissue degradation is rapid in oysters after death. Therefore, tissue collection during the field trials was rarely achieved. It was not possible to definitively confirm the cause of oyster death as *Bonamia* sp. However, based on the history of the site with previously low mortality rates and the recent occurrence of the first outbreak of clinical infection, at the time of the study it was most likely that deaths were predominantly due to active *Bonamia* sp. infection. The analysis of results from the field trials focused on determining risk factors significantly associated with oyster death on that site. These results were considered of value to oyster farmers as broadly representative of management practices that may reduce oyster death. The results also informed purported risk factors in the design of the tank trials where oysters

could be checked repeatedly, and tissue recovered and tested for *Bonamia*. Thereby, risk factors associated with the progression from subclinical to clinical bonamiosis in Native Oysters were informed from the field trials but determined specifically from the two tank trials.

Calculation of the optimal qPCR cycle threshold was completed using two methods: applying histopathological examination as a gold standard test (i.e. with a specificity and sensitivity of 100%) and applying a latent class method (i.e. assuming a lack of a gold standard test). The results of both methods are presented in this report, but the latter result was used to confirm the *Bonamia* sp. infection status of study oysters. This classification informed an analysis of risk factors significantly associated with the conversion from subclinical to clinical bonamiosis.

Source farm and oyster selection

Oysters from all Victorian trials were sourced from one of three established farms in three distinct geographic locations in Port Phillip Bay and Western Port Bay, Victoria (Figure 4). Farms were selected based on a known history of testing for *B. exitiosa* and, in one case, the farmer-reported recent incidence of oyster mortalities (see Appendix 1) for a description of collated farm-level information prior to the Victorian project start). Farm 1 oysters were sourced from a coastal lease at the Grassy Point Aquaculture Reserve located on the western side of Port Phillip Bay (38° 6.226' S, 144° 41.256'E), with evidence of mortalities purportedly due to a high prevalence of *B. exitiosa* infection over several years (see Results). Farm 2 oysters were derived from a land-based hatchery facility in Western Port Bay (38° 28.049'S, 145° 2.324'E) where *Bonamia* sp. infection had been diagnosed, but there was no evidence of deaths due to clinical bonamiosis. Oysters within Farm 3, from the eastern side of Port Phillip Bay (38° 14.162'S, 145° 0.481'E) had been tested for *Bonamia* sp. annually with universally negative results.

Figure 4. Geographic location of source Farms (1, 2 and 3) from which oysters were sourced for two tank trials assessing risk factors for clinical bonamiosis in farmed Native Oysters (inset: Victoria, Australia, with location of Port Phillip Bay and Western Port

Field Trials

Field Trial 1 (2016)

Field Trial 1 took place at Farm 1 (Grassy Point Aquaculture Reserve) where there was a history of both subclinical and clinical bonamiosis. This farm operated with grow-out baskets that were 650 mm long with 20 mm mesh and attached to long lines with "dropper" ropes spaced approximately 1m apart. The study design was a 2x3 factorial design nested within a randomised block design. There was also a control site on Farm 3 (i.e. no history of *Bonamia* sp. infection) where oysters were held under different conditions to Farm 2. The unit of interest was the individual oyster. The trial took place between February 2016 and July 2016.

There were three risk factors of interest: size of oyster (indicative of age <40 mm versus >56 mm), stocking density in the basket (either 33% filled with oysters or 66% filled with oysters) and depth of the basket in the water column (2m deep versus 7m deep). These risk factors were identified after discussion with Victorian oyster farmers as factors that they could be manipulated within their production systems. Further details about Farm 1 and the management practices can be found in Appendix 1.

All oysters on Farm 1 were brought ashore and double-graded, sorted and placed into different treatment baskets. The allocation of treatments to baskets (and position on the long line) was undertaken using a random number generator. The total of the

remaining farm stock was counted into baskets (4,627 oysters). A large proportion of oysters were discarded as mortalities during the sorting process.

Thirty-eight study baskets, containing between 35 and 279 oysters, were submerged onto one of two horizontal longlines on 12th February 2016. Each basket was assembled based on a combination of the three potential risk factors, with each combination replicated between three and seven times. Six baskets, assembled to mimic "normal" risk factor levels on Farm 1 (i.e. mixed size oysters, 50% basket density and a water depth of 5 metres) were included in the trial as "control" baskets. These contained between 80 and 101 oysters. On the 19th of August 2016 (189 days after stocking) baskets were pulled out of the water and oysters counted and sorted into "dead" and "alive". The mortality rate was calculated as the number of oysters confirmed as dead divided by the total number of oysters in the basket. Basket allocations are displayed in Table 5.

Table 5. Basket allocations for the first field trial

Number of baskets (with combination of factors)	***Oyster age (surrogate measure of size)***	***Oyster density (% basket filled)***	***Oyster depth in sea water (m)***
5	Large	30	2
7	Large	30	7
6	Large	60	2
4	Large	60	7
4	Small	30	2
5	Small	30	7
3	Small	60	2
4	Small	60	7

Water quality parameters (e.g. temperature, phytoplankton levels) were measured and recorded throughout the trial (Hydro Cat EP, Sea Bird Electronics).

Analysis

Significant associations between the assessed risk factors, both individually and in two- and three-way interactions, and the basket-level mortality rate over the period of the study was assessed using a generalised linear mixed model within the nlme package in RStudio (RStudio Team (2016); RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/), including line as a random effect. Results were presented as odds ratios and significance indicated using *p*-values.

An interaction term allows testing of the hypothesis that the relationship between one risk factor and the outcome is different at varying levels of another risk factor. If an interaction term is significant, it indicates the effect of one risk factor on the outcome is significantly different at different values of the other risk factor.

Field Trial 2 (2017)

Field Trial 2 was also conducted on Farm 1 and commenced on the 2nd of February 2017 and was concluded on the 9th of April 2018. The study design was a 2x2 factorial design nested within a randomised block design. The control site remained, as previously, on Farm 3 (i.e. no history of *Bonamia* sp. infection). The unit of interest was the individual oyster.

There were two risk factors of interest: degree of fouling of the baskets holding the oysters (heavy and light fouling) and the stocking density in the basket (either 33% filled with oysters or 66% filled). The degree of fouling was a qualitative measure controlled by the level of cleaning of the baskets when they were removed from the water for monitoring. The "clean" baskets were carefully hosed using the on-board high-pressure hose until the bulk of the fouling organisms were removed. The control baskets underwent moderate cleaning and the "dirty" baskets were not cleaned. There were 16 baskets in total in the trial with four baskets allocated as controls on the site. The control baskets were stocked approximately half full and given a light clean when monitored during the trial. In total there were 925 oysters in the trial with between 30 and 107 oysters stocked into an individual basket. Oysters within these baskets were of mixed size and year class, ie they were not graded prior to the trial commencing. All baskets were clean at the beginning of the trial and were the same baskets that were used in Field Trial 1. The allocation of treatments to baskets (and position on the long line) was undertaken using a random number generator.

Trial baskets were cleaned, checked and photographed on the 29th May 2017, 12th of October 2017 and 9th of April 2018.

Tank Trials

Tank Trial 1 (2016)

Independent, controlled tank trials were conducted in two sequential years at the same facility (Marine and Freshwater Discovery Centre; Victorian Fisheries Authority; Queenscliff; Victoria; Australia) to investigate risk factors significantly associated with the conversion of a *B. exitiosa* infection from subclinical to clinical within Native Oysters.

Tank Trial 1 commenced on the 27th of February 2016. The unit of interest was the individual Native Oyster (*O. angasi*). Live farmed oysters were selected from a range of baskets over two long lines within Farm 1 (Grassy Point) and immediately graded, resulting in a final trial population of 240 oysters with a mean size of 67 mm. Ten oysters were held in modified SEAPA® baskets within each of 24 individual 100 litre tanks for the trial period of 56 days. Seawater was supplied to the tanks from Port Phillip Bay at a flow rate of 100 ml per 10 seconds.

Each possible combination of three risk factors, namely water temperature (i.e. warm versus ambient), water motion (none versus oscillation) and nutrition (starved versus

adequate nutrition), were applied across multiple tanks in a 2^3 (i.e. 2 x 2 x 2) factorial design nested in a randomized control block design (RCBD) (Figure 5). Due to a lack of adequate space at the facility, a single replicate negative control tank was applied during Tank Trial 1, with ten oysters sourced from Farm 3. A single positive control tank was also applied with ten oysters sourced from Farm 1 held at ambient temperature and with adequate nutrition for the duration of the trial.

Ambient water was supplied direct from Port Phillip Bay and "warm" water was heated by an Aquahort heat pump (Model AH2000HO). Temperatures were recorded by four in-tank thermometers and recorded daily from one thermometer in each of the ambient and heated tanks. The mean ambient temperature was 18.2°C C and the mean heated temperature was 22.9°C. Oysters in the "adequate nutrition" group were fed three times per week at a rate of 5 ml per 15 seconds. The feed was algae harvested from a Seawater Continuous Algal Production System (SEACAPS) stored in 5000L open air tanks enclosed in a greenhouse environment. The species composition was approximately 50% *Chaetoceros muelleri*, 27% *Pavlova lutheri* and 23% *Tisochrysis lutea*. All species stocks were originally sourced from the Australian National Algal Supply Service at CSIRO in Tasmania. All species were fed on f2 nutrient media.

'Starved' oysters were not fed during the entire trial period of 56 days. Tumbling was simulated using an SMI Multi-tube vortexer oscillator (Analytical instruments LLC) at 1700 rpm with each group of oysters being oscillated in a group specific container for 60 seconds every second day. All tanks were cleaned every second day and strict biosecurity was maintained between tanks.

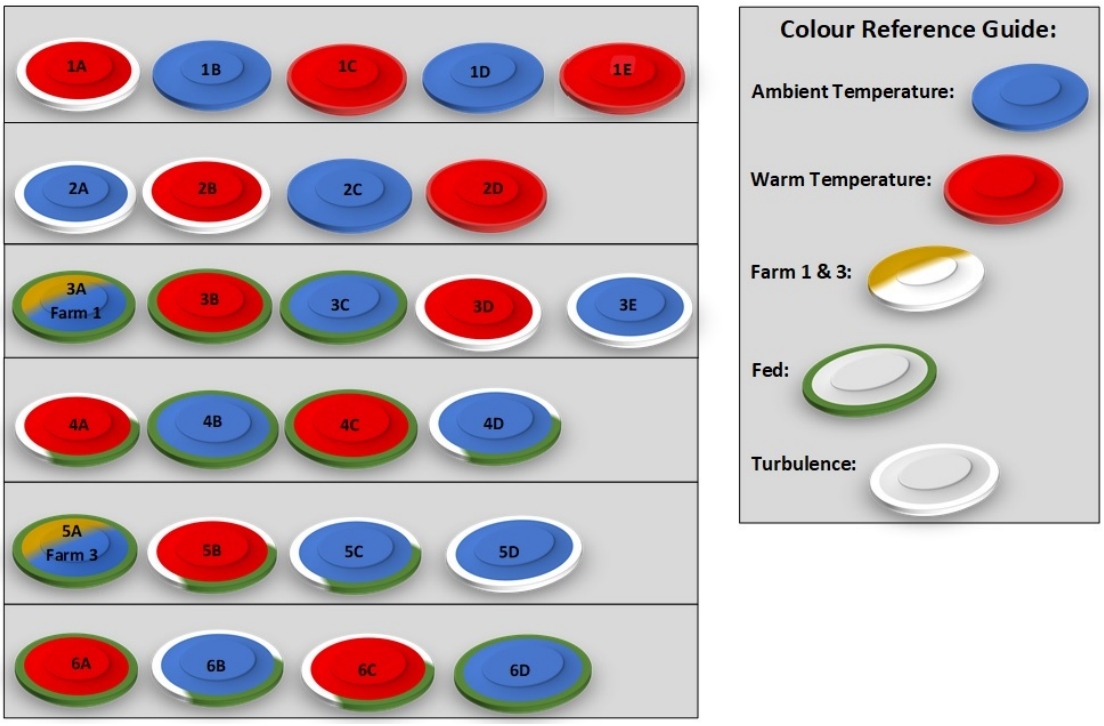

Figure 5. Layout of Tank Trial 1 assessing potential risk factors for inducing clinical bonamiosis disease in sub-clinically infected *O. angasi*

Tank Trial 2 (2017)

Tank Trial 2 commenced on the 23th of March 2017. Similar to Tank Trial 1, each possible combination of three risk factors were applied in triplicate in a 2^3 factorial design nested in a RCBD, namely: source (Farm 1 versus Farm 2), oyster size (small; 52 – 85 cm versus large; 74 – 154 cm) and stress (high temperature and starved versus ambient temperature and adequately fed; as for Tank Trial 1) for a total trial period of 83 days (Figure 6). These risk factors were selected based on the results of Tank Trial 1 and Field Trial 1. Ten sampled oysters were held within modified baskets in each of 24 individual 100 litre tanks for the duration of the trial. Additionally, forty oysters from Farm 3 were evenly distributed across four negative control tanks.

In both years, Tank Trial oysters were checked at least once daily by a trained technician to monitor and collect tissue from dead oysters. Checking involved observing whether oysters were open (and feeding). If there was concern about the status of the oyster in terms of dead or alive then the oyster was tapped with a knife and an attempt made to gently pry open the two shell halves to gauge resistance. However, despite consistent monitoring, tissue could not always be recovered as tissue degradation was rapid within dead oysters. All other features of the tank set-up, such as feeding and water temperature, were maintained over the two tank trials.

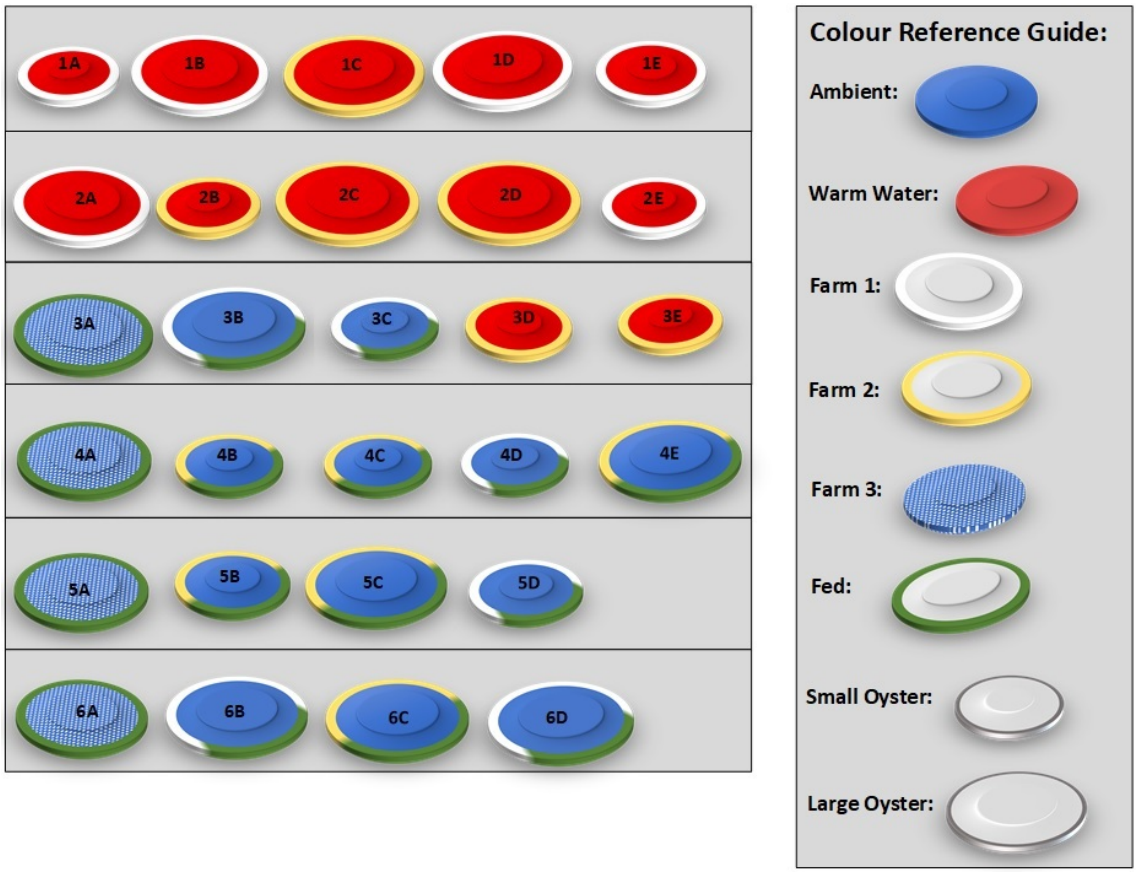

Figure 6. Layout of Tank Trial 2 assessing potential risk factors for inducing clinical bonamiosis disease in sub-clinically infected *O. angasi.* (Farm 3 = negative control)

Diagnostic tests

In 2016, tissue from trial oysters (n=250) were, where possible, collected and tested for *B. exitiosa* using a qPCR. In 2017, tissues from trial oysters (n=280) were, where possible, collected for both qPCR and histopathological examination.

All qPCR testing was undertaken at the Commonwealth Scientific and Industrial Research Organisation (CSIRO; Geelong; Victoria; Australia). The *Bonamia sp.* TaqMan qPCR (Corbeil et al., 2006) that amplifies partial SSU rRNA previously referred to in this report was utilised to detect *Bonamia* sp. A small piece of gill was taken from each oyster and fixed in 95% ethanol as per OIE recommendations (OIE, 2017). Each sample was tested using the *Bonamia* sp. qPCR, with the 18S rRNA qPCR applied as the positive extraction control. All samples that did not produce a C_T value of >0.1 after 45 cycles were considered negative. The mean of two C_T values was used where both wells were positive. An indeterminate qPCR result was applied when one of the duplicates produced a positive result and the other a negative result indicating that the level of target in this sample was close to the limit of detection for this test (see relevant section in this report for information on the qPCR).

One or more section(s) of tissue were taken from each oyster, including the mantle, gills, digestive gland and/or gonad and fixed in 10% buffered formalin for a minimum of 24 hours (OIE, 2017). Where the oyster was sufficiently small or limited tissue remained, the whole oyster was placed in fixative after the PCR sample was removed. A 3mm wide section from each available tissue was trimmed, embedded in paraffin and 5-micron sections were cut and stained in haematoxylin and eosin using standard methods (Centre for AgriBioscience; Bundoora; Victoria; Australia). All histopathological examination of slides was undertaken by an experienced, senior pathologist. Sections for examination were selected at random and assessed with no reference to observations on other sections from the same oyster. Each section was scanned at low power (x 40) to determine:

- The quality of fixation and staining and general orientation of the section(s);
- The overall condition of the oyster, including abundance of Leydig cells and presence of ingesta in stomach and intestine;
- The presence and location of focal or generalised inflammatory changes; and
- The absence of specific tissues.

Subsequently, each tissue was examined at higher power (x 100, x 400) to determine the nature, intensity and distribution of inflammatory and degenerative changes and presence or absence of *B*. *exitiosa*. Areas were targeted where focal or generalised inflammatory changes were observed in specific tissues, using oil immersion (x 1000) as required. Identification of *B. exitiosa* followed the Australia and New Zealand Standard Diagnostic Procedure (Corbeil et al., 2006). The occurrence, distribution and number of *B. exitiosa* observed in tissues was recorded in a semi-quantitative manner based on previous work by Diggles (Diggles, Cochennec-Laureau & Hine, 2003) and Johnston (Johnston, 1992) but with further refinement. The classification system developed provided a mechanism whereby the severity and distribution of

inflammatory or degenerative responses could be assessed in a semi-quantitative manner across organs and tissues. This was particularly important given that many infected haemocytes or free organisms could be missed, or intact haemocytes were not fully represented.

Descriptive analysis
Data was recorded in a spreadsheet using Microsoft Excel (2016). The amount of *B. exitiosa* observed within each histopathological section was categorised according to the following (Table 6).

Table 6. Classification of *Bonamia sp.* occurrence in histopathological sections

Description of infection level	Category
Not evident in section	0
Single or occasional *B. exitiosa* observed	1
Low numbers observed (<10 per high power field)	2
Moderate numbers observed (10 – 100 per high power field)	3
High numbers observed (>100 per high power field	4

Sections with single or occasional *B. exitiosa* observed were categorised as 1. An oyster-level binary score of *Bonamia* -positive, based on histopathological examination, was also assigned even if only a single or occasional *B. exitiosa* (category 1) was visualized in at least one tissue section. In several cases, bodies that were not morphologically typical of *B. exitiosa*, possibly due to autolysis or degradation, but which could not be absolutely excluded as being *B. exitiosa*, were assessed as equivocal (+/-). These cases were excluded from further analysis.

A minimum of 10-15 high power fields were examined for each tissue component of each oyster examined. This followed an overview of the different tissue components of each section at low power (x40 / x 100). In cases where a clear haemocytosis was evident at low power, high power assessment was easily made for each tissue component. In cases where few or occasional *Bonamia* sp. were seen, high power examination focussed on these areas. In cases where no *Bonamia* sp. were evident on low power examination, each tissue component in the section was scanned at high power for evidence of *Bonamia* sp. The numbers of *Bonamia* sp. identified are a semi-quantitative evaluation of the section and reflect an approximation of the overall average of organisms present.

Estimation of diagnostic test sensitivity and specificity

The performance of histopathological examination and qPCR was assessed using a latent class model with a Bayesian approach (Branscum et al., 2005), with data derived from Tank Trial 2. This allowed calculation of the sensitivity (Se) and specificity (Sp) of the two tests, as well as the prevalence in two Farms (1 and 2) from a number of assumptions. It was assumed that the two tests were conditionally independent (i.e. the Se and Sp of qPCR were independent of the outcome of histopathological

examination when used to test the same individuals) and that the proportion of truly infected oysters differed between Farm 1 and Farm 2. It was also assumed that diagnostic test properties were constant across both Farms.

Within a Bayesian analysis, prior distributions of all parameters, including sensitivity, specificity and prevalence, must be specified and reflect known information. The prior distributions for the Se and Sp for histopathological examination and prevalence of *Bonamia* sp. in Farm 1 and 2 were modelled as Beta (a, b) distributions. The specific shape parameters a and b were derived based on the most likely value (mode) and *n*th percentile of the values found in the literature and based on expert opinion (Table 7). Prior information on the Se and Sp for the qPCR tests were modelled using the Beta (1, 1) distribution which is uniform for the interval between zero and one (i.e. an uninformative prior). All Beta prior distributions were parameterised using epi.betabuster in RStudio.

Table 7. Distributions for prior information of known variables for diagnostic tests of *Bonamia sp.* in Australian Native Oysters (Se = sensitivity; Sp = specificity; qPCR = quantitative polymerase chain reaction; histo = histopathological examination)

Parameter	Most likely value (mode)	Percentile	Percentile's value	Beta distribution	Reference
Sp – histo	0.999	20	0.90	Beta(15.47,1.01)	(Diggles et al. 2003)
Se - histo	0.440	20	0.35	Beta(8.57, 10.64)	(Diggles et al. 2003)
Prev – P1	0.172	20	0.10	Beta(1.73, 4.50)	Trial 2 trial
Prev – P2	0.390	20	0.30	Beta(6.59, 9.74)	Trial 2 trial

The analysis was implemented in OpenBUGS version 3.2.3 rev 1012, using a Markov Chain Monte Carlo (MCMC) sampling algorithm to obtain a Monte Carlo (MC) sample from the posterior distribution (Thomas et al., 2006). The first 10,000 iterations were discarded as a burn-in to allow convergence and the successive 40,000 iterations were used for the posterior inference. Convergence was assessed by visual inspection of the time-series plots of selected variables, as well as Gelman-Rubin diagnostic plots using three sample chains with different starting values (Toft et al., 2007). Posterior inference was performed by calculating the median and 95% posterior credible intervals (95% PCI, the Bayesian analog of a 95% confidence interval) of the Se and Sp of the two tests, as well as the prevalence in Farms 1 and 2.

To investigate whether the specified prior knowledge would have affected the parameter posterior estimates, the analysis was repeated using uninformative priors for all parameters, as well as different levels of certainty in the definition of priors for Se and Sp of qPCR and histopathological examination. The models with different priors were compared using the deviance information criteria (DIC), with the best model reported (i.e. smallest DIC) (Spieglhalter et al., 2002).

Calculation of optimal qPCR cycle threshold (C_T)

Two methods were applied to determine the optimal qPCR cycle threshold (C_T), namely, applying histopathological examination as a 'gold standard' test (i.e. assumed 100% sensitivity and specificity) and applying a latent class method, where there is an assumption that no gold standard test is available.

i. Histopathological examination applied as the 'gold standard'

A Receiver Operating Characteristic (ROC) analysis was used to determine the optimal cycle threshold for the qPCR to maximise accuracy relative to histopathological examination. The area under the curve (AUC) of a ROC curve indicates the ability of a test to correctly classify those with and without infection. An AUC of 0.5 indicates a test has the minimum discriminatory ability (i.e. is equivalent to tossing a coin).

ii. Latent class analysis

To determine the optimal epidemiological cycle threshold value of the qPCR, separate Bayesian analyses were performed with an increase from ≤25 to ≤40, in 1 value increments, with histopathological examination and prevalence data kept constant. A two-graph receiver operating curve plot (sensitivity-specificity plot) was constructed to illustrate the change in qPCR sensitivity and specificity with a decrease in C_T value, with the optimal epidemiological cut-point defined as the point at which the Se and Sp crossed (i.e. both parameters were maximized).

Logistic regression analysis - Risk factors for clinical bonamiosis

Oysters in Tank Trials 1 and 2 were then categorised into four subsets based on a qPCR C_T value at or below the optimal cut-point and whether the oyster had died at trial end (Table 8).

Table 8. Parameters for categorisation of Native Oysters, based on an optimal epidemiological qPCR CT value cut-point and dead/alive status at trial end

Category	**qPCR C_t value**	**Dead/Alive at trial end**
Clinical bonamiosis	≤ optimal cycle threshold	Dead
Subclinical bonamiosis	≤ optimal cycle threshold	Alive
Death due to reason other than *Bonamia* sp. infection	> optimal cycle threshold	Dead
Non-infected	> optimal cycle threshold	Alive

Risk factors with a *p*-value of <0.20 in univariable logistic regression were evaluated using generalised linear mixed models within the nlme package in RStudio (RStudio

Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/), with separate models created for both the trials. To determine risk factors for the development of clinical bonamiosis, oysters categorised with 'clinical bonamiosis' were compared against oysters with 'subclinical bonamiosis' (see Table 8 for definitions). Oysters in other categories were excluded from further analyses. Model building used manual backwards elimination, with all eliminated factors re-evaluated for confounding effects and biologically meaningful pairwise interactions assessed between the final model variables. An effect of clustering at the tank level was evaluated in the final model using tank as a random effect. Model fit diagnostics were evaluated (Lemeshow & Hosmer, 1982). Results were presented as odds ratios and significance indicated using *p*-values, with statistical significance set at $p<0.05$.

Survival analysis – risk factors for time to clinical bonamiosis

Median survival time of *Bonamia*-positive oysters to death (clinical bonamiosis) was estimated using the Kaplan-Meier method along with finite sample pointwise confidence intervals and 95% Hall-Werner confidence bands (Hall & Wellner, 1980), with differences between categories evaluated by the log-rank test. Explanatory variables with $p<0.20$ in univariable Cox proportional hazards regression models were assessed using multivariable Cox modelling with robust standard errors calculated to account for within-tank clustering. Model fitting used a manual backward elimination approach, with significance set at $p<0.05$. The proportionality assumption was tested using Schoenfeld and scaled Schoenfeld residuals and the fit of the final model to the data was checked using Cox-Snell residuals. Results were presented at Hazard ratios and significance indicated using p-values, with statistical significance set at $p<0.05$.

qPCR testing for other jurisdictions

Limited numbers of oysters were selected from a farm in NSW and Tasmania to screen for the presence of *B. exitiosa.* Samples were selected by the farmer in NSW and project staff from a range of areas on the farm in Tasmania. All samples were mature (older than 2 years) *O. angasi.*

4.3 Results

Descriptive findings related to the contemporary farming practices and bonamiosis outbreak prior to the commencement of the project are provided in Appendix A.

Field Trial 1

Water parameter measurements

Hourly data for the following parameters were collected from the logger deployed at Farm 1, with additional comparative data collected from a logger at a pristine site in central Port Phillip Bay. The parameters available were temperature, depth, salinity, dissolved oxygen and chlorophyll a (fluorescence).

1. *Temperature*

No significant features (Figure 7).

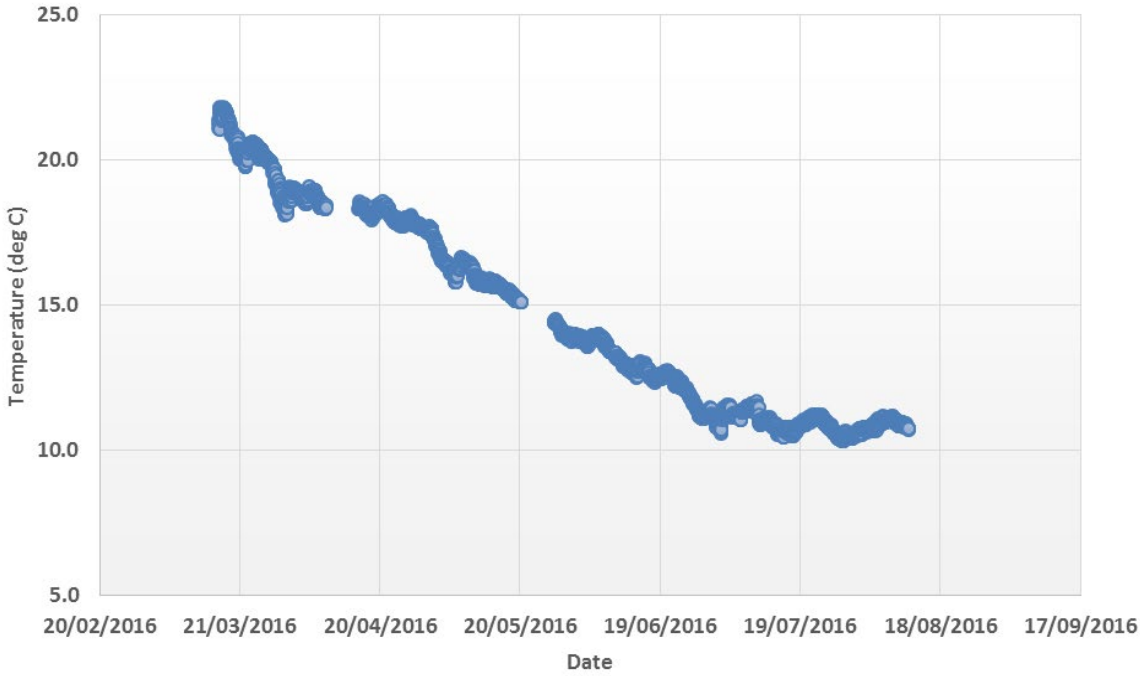

Figure 7. Temperature (degrees C) for Farm 1 (Grassy Point; Port Phillip Bay) during the Field Trial 1 study period (February 2016 to July 2016)

2. *Salinity*

The only significant source of salinity into Port Phillip Bay is Bass Strait, with river and sewage in-flows essentially fresh. The salinity of Bass Strait is 35.0-35.5 parts per thousand, with the salinity in Port Phillip Bay relatively less due to mixing of freshwater with seawater. However, evaporation (particularly in the shallow Geelong Arm) can lead to salinity higher than 35 ‰. During the drought in 2008, salinity of up to 38 was observed. At the time of the Field Trial 1, the salinity was greater than Bass Strait, but less than during the recent drought, with a decline due to runoff from the (average) wet winter (Figure 8).

3. Dissolved oxygen

Dissolved oxygen is supplied from the atmosphere, and from photosynthesis, and is consumed by microbial breakdown of organic matter in the water column and sediment. The recorded data during Field Trial 1 was in the “normal” range of ~90-100%, with peaks in mid-June, mid-July and early August correlating with chlorophyll peaks (i.e. phytoplankton primary production). Similarly, the low values in the second half of the record may have related to the decomposition of the algal blooms (Figure 9).

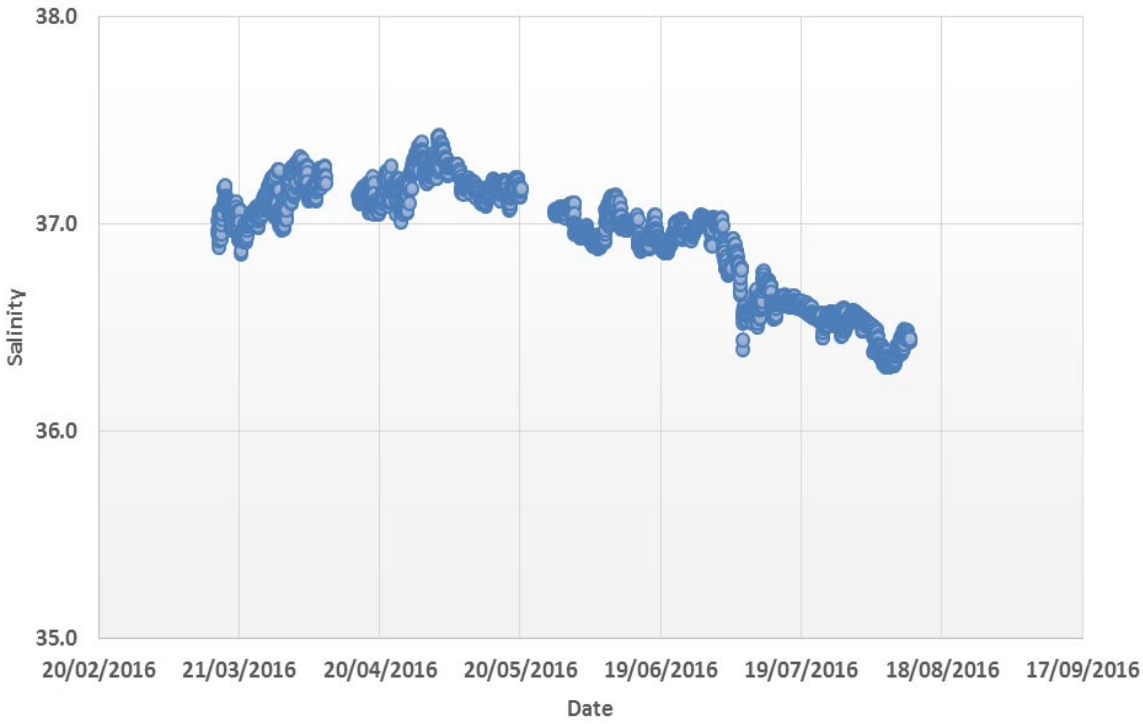

Figure 8. Salinity (parts per thousand) for Farm 1 (Grassy Point; Port Phillip Bay) during the Field Trial 1 study period (February 2016 to July 2016)

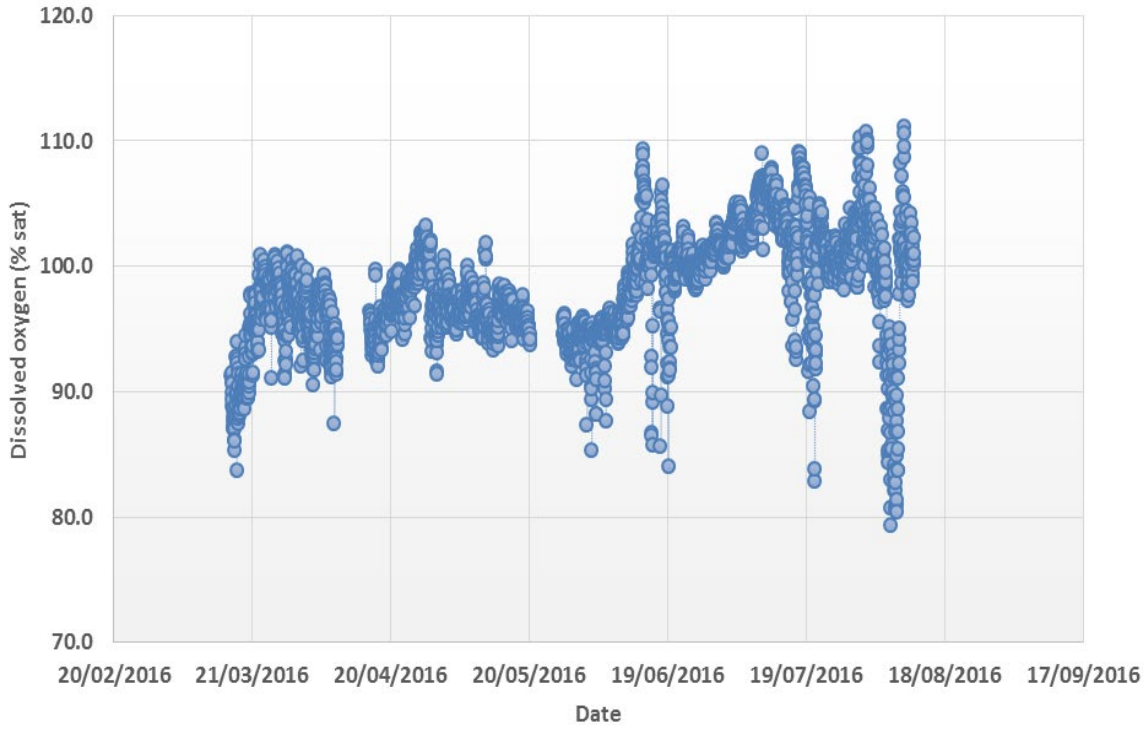

Figure 9. Dissolved oxygen (% saturation) for Farm 1 (Grassy Point; Port Phillip Bay) during the Field Trial 1 study period (February 2016 to August 2016)

4. Chlorophyll

Chlorophyll is the pigment plants use to capture sunlight during photosynthesis. There are a range of different pigments that plants have used to adapt to different light regimes, but chlorophyll *a* is the most abundant. Chlorophyll fluoresces when exposed to light of a particular wavelength, and the automated detection of chlorophyll fluorescence is based on the hypothesis that the amount of fluorescence is related to the amount of chlorophyll (which in turn is related to the abundance of phytoplankton in plankton-dominated systems). The relationship between fluorescence and chlorophyll is not constant, but varies with phytoplankton species, nutritional status and light history.

In Port Phillip Bay, chlorophyll is often elevated (4-10 µg/L) near the major nutrient inputs, specifically the Yarra and Western Treatment Plant (WTP), and lowest (<2 g/L) everywhere else. The peaks in the data at 13 June, 15 July, 31 July and 11 August all indicate winter phytoplankton blooms. The blooms are short-lived, and whilst there are no comparative data from this site, the size and frequency of the blooms (4 in 6 weeks) was unusual. Winter is the preferred season for *Rhizosolenia* blooms, but there was no analysis of phytoplankton identification data (though samples have been collected from nearby sites for other programs). Modelling has indicated that the WTP plume can reach this area on occasion, and the blooms may have been initiated by the WTP plume blowing toward the Geelong Arm, which collapsed after wind direction changed, shifting the plume, and the plankton used up the remaining nutrients (Figures 10 and 11).

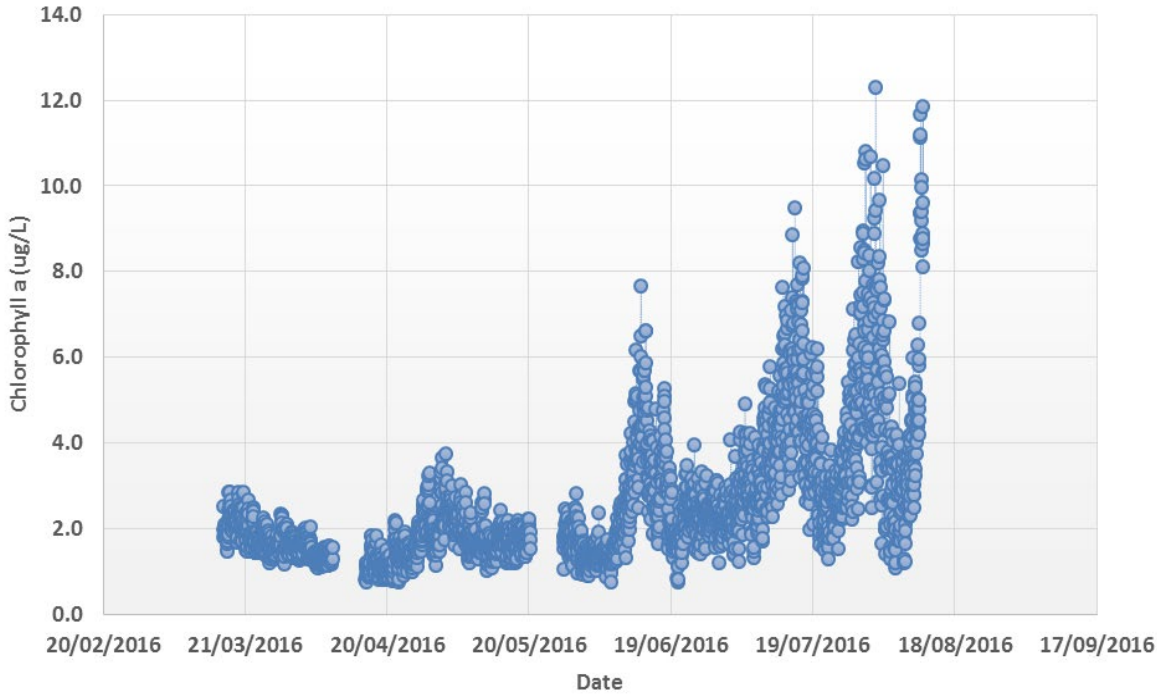

Figure 10. Chlorophyll a (µg/L) for Farm 1 (Grassy Point; Port Phillip Bay) during the Field Trial 1 study period (February 2016 to July 2016)

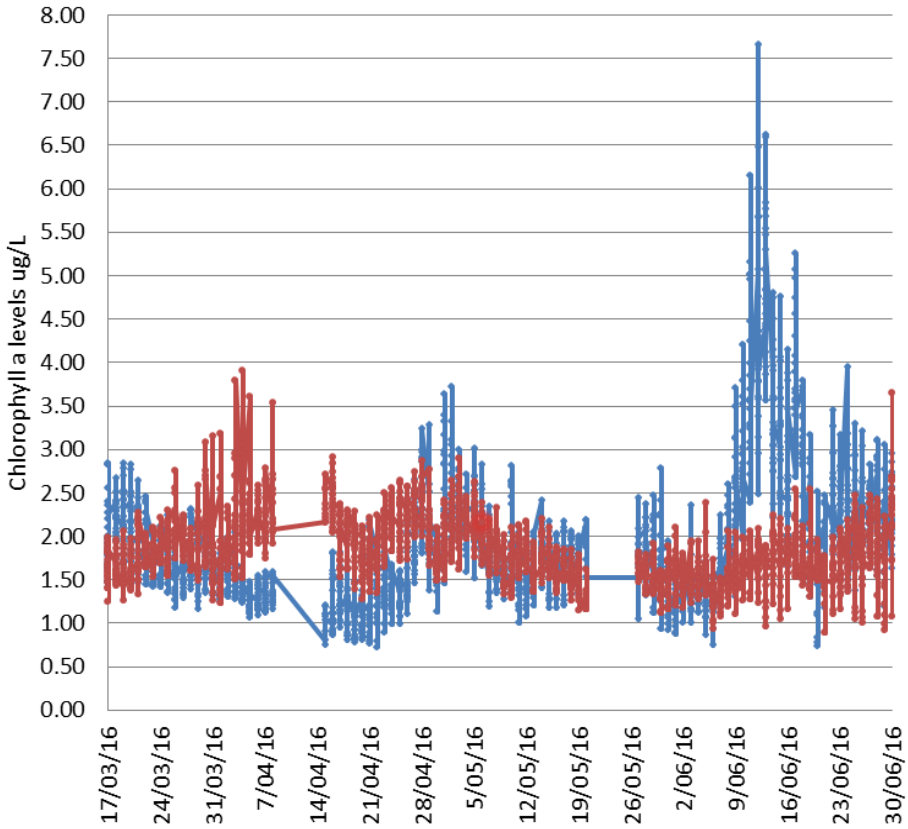

Figure 11. Comparison of chlorophyll a fluorescence (µg/L) for Farm 1 (Grassy Point; Port Phillip Bay; blue) and central site (Port Phillip Bay; red) during the Field Trial 1 study period (February 2016 to July 2016)

Risk factors associated with basket-level mortality rate (oyster status: dead versus alive)

The mean mortality rate of 0.35 ± 0.04 within the Farm 1 control baskets (mixed size oysters at 50% density and 5 metre depth) was higher than the mean mortality rate experienced in trial baskets (0.30). This indicates that some combinations of factors (e.g. large oysters at 30% density and 2 metre depth; mortality rate = 0.25 ± 0.10) may be protective against oyster losses. In contrast, other combinations (e.g. small oysters at 60% density and 2 metre depth; mortality rate = 0.50 ± 0.04) were potentially imposing increased stress on the oysters, resulting in a higher mortality rate.

Table 9. Field Trial 1 - Mean, minimum and maximum number of oysters within baskets of each combination of risk factor at the trial start date and mean mortality rate (± standard deviation; SD)

Risk factors			Number of oysters			Mortality rate (%)	
Oyster size	**Density**	**Depth**	**Mean**	**Min**	**Max**	**Mean**	**SD**
Small	**30%**	**2 metres**	104	100	113	0.39	0.07
		7 metres	114	100	125	0.36	0.11
	60%	**2 metres**	253	220	278	0.50	0.04
		7 metres	237	220	279	0.38	0.03
Large	**30%**	**2 metres**	43	35	50	0.25	0.10
		7 metres	48	39	60	0.32	0.15
	60%	**2 metres**	90	75	100	0.31	0.11
		7 metres	86	75	92	0.29	0.04
Control baskets (mixed size)	**50%**	**5 metres**	89	80	101	0.35	0.04

There appeared to be considerable variation in basket-level mortality rates between each combination of the risk factors (Table 9, Figure 12). However, within both the univariable and multivariable models, only oyster size was significantly associated with this outcome (Figure 13). On average, small oysters had a 9.9% higher mortality rate relative to large oysters (p <0.01). At the basket-level, depth in the water and basket density, both individually and as interaction terms, were not significantly associated with basket-level mortality rate.

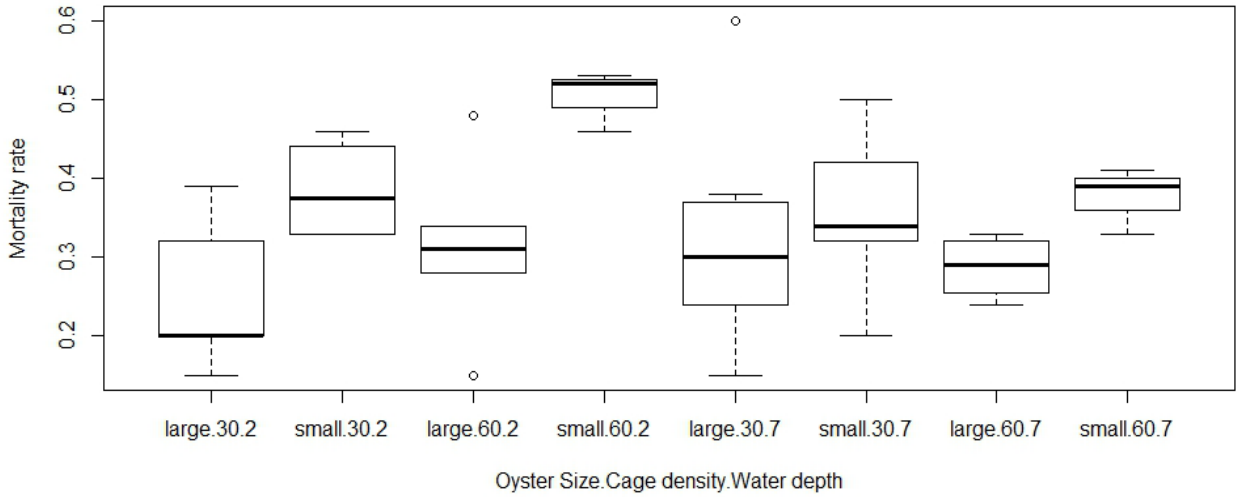

Figure 12. Basket-level mortality rate of Native Oysters by available three-way combinations of potential risk factors: Oyster size (small vs large); oyster density in basket (30% vs 60%) and water depth (2 m vs 7 m) in Field Trial 1

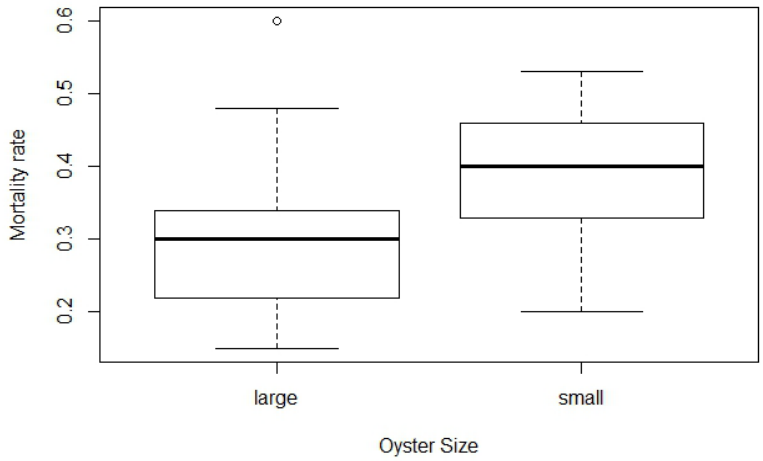

Figure 13. Basket-level mortality rate, by Native Oyster size alone (small vs large) in Field Trial 1

Field Trial 2

The mean mortality rate within the four control baskets of Farm 1 (intermediate density and moderate level of cleaning) and the treatment baskets was not significantly greater or less (p>0.20), regardless of the combination of basket cleanliness and density applied (Table 10).

Table 10. Field Trial 2 - Mean, minimum and maximum number of oysters within baskets of each combination of risk factor at the trial start date and mean mortality rate (standard deviation; SD) as at 9 April 2017

Risk factor		Number of oysters			Mortality rate	
Density	**Cleanliness**	**Mean**	**Min**	**Max**	**Mean**	**SD**
Dense	**Clean**	73	70	78	0.44	0.05
	Dirty	95	80	106	0.47	0.16
Not dense	**Clean**	32	30	35	0.42	0.18
	Dirty	33	32	35	0.55	0.04
Control		54	40	69	0.43	0.21

Tank Trials 1 and 2

Descriptive analysis

A total of 490 oysters sourced from Farms 1 and 2 were sampled across both trials. Twenty-three oysters (Tank Trial 1) and eight oysters (Tank Trial 2) died and did not have tissue available for testing by qPCR. At the end of both trials, 29.2% of the available 459 oysters had a C_T value of ≥45.0 (analytical cut-point). The median C_T value of the remaining oysters, by farm was 29.8 (Farm 1; n=228) and 22.9 (Farm 2; n=97) (Figure 14).

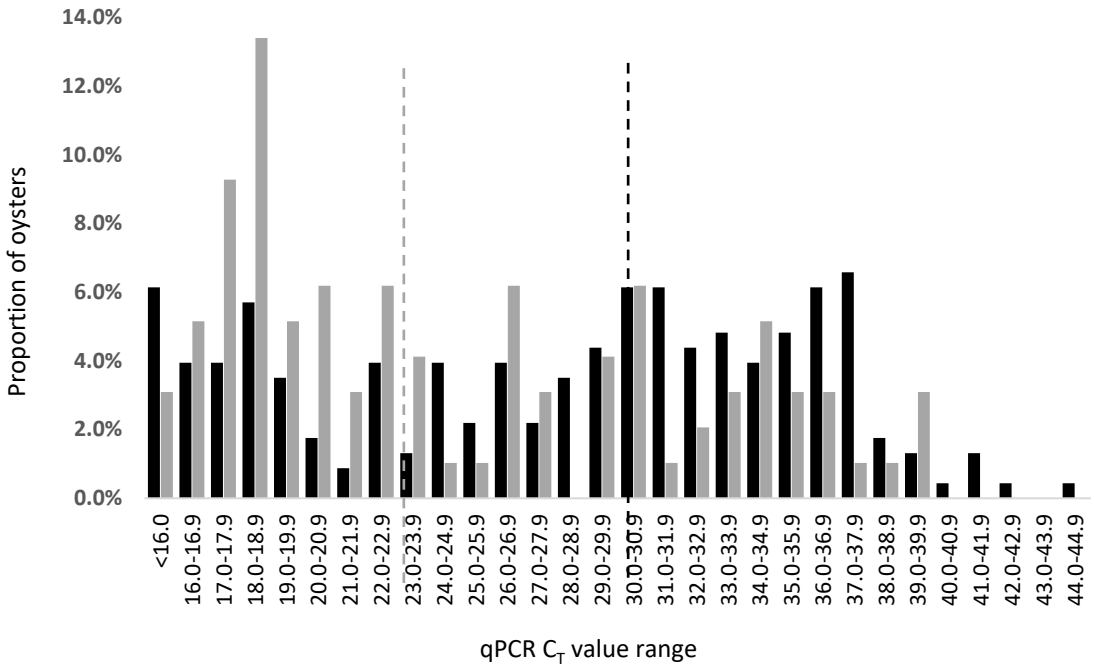

Figure 14. Proportion of farmed Native Oysters that were diagnosed with a *Bonamia* qPCR cycle threshold (C_T) value of <45.0, at intervals of 1.0 (dashed line = median C_T value), stratified by source farm (Farm 1 (n=228): black; Farm 2 (n=97): grey). Dotted areas = median C_T value

Tissue from seven oysters within Trial 2 were unavailable for histopathological examination. Of the remaining oysters, the majority (222 oysters; 92.5%) had five or six tissue sections (interstitium, stomach, digestive gland, intestine, gills and/or palp) available for examination, with the interstitium and digestive gland most commonly sampled (98.3%-99.6%).

Bonamia exitiosa was observed most commonly and in the highest numbers within the interstitium, digestive gland and palp and least commonly in the stomach (Figure 15). Of the 108 oysters diagnosed as *Bonamia*-positive based on histopathological examination, 80% were based on examination of the interstitium and/or digestive gland.

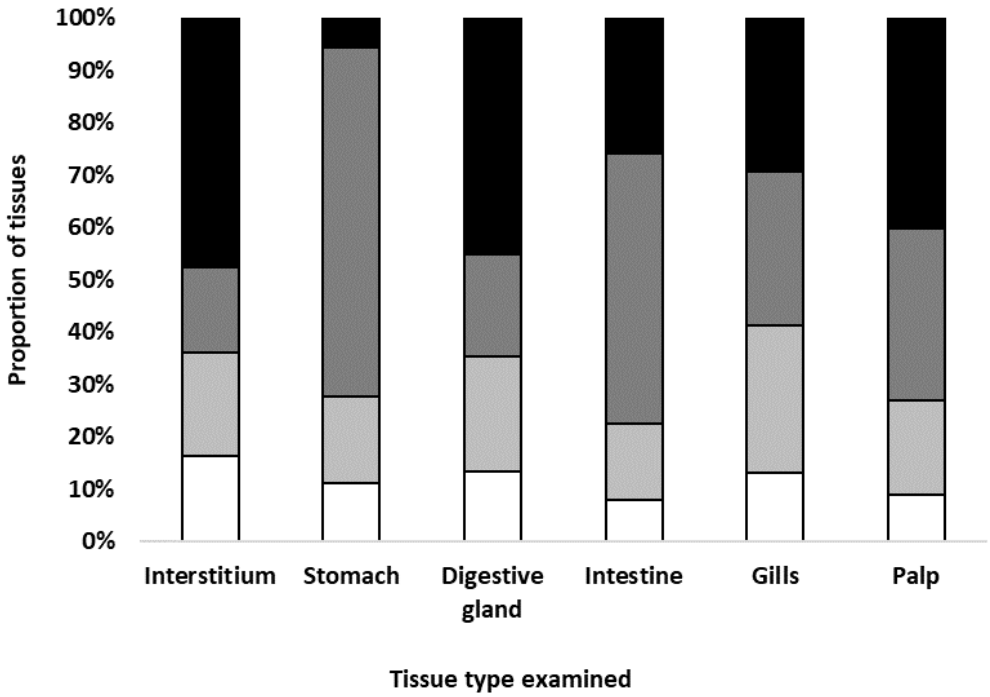

Figure 15. Proportion of *Bonamia*-positive tissue sections (n = 18 to 86) that had single/occasional (white), low (light grey), medium (dark grey) and high (black) numbers of *B. exitiosa* observed by tissue type, in farmed Native Oysters

Bonamia sp. was observed in four or more tissues within 82.9% of infected, dead oysters, compared to 18.9% of infected, live oysters, indicating dissemination of the parasite throughout an oyster was associated with death.

In total, 47 of 50 Farm 3 oysters (i.e. negative controls) (94.0%) were alive and *Bonamia*-negative on qPCR and histopathological examination at the end of the trial period (across Tank Trials 1 and 2). This indicated that the overall tank environment (considered independently of any disease condition and/or stress) did not significantly influence oyster survival during both trials. Two Farm 3 oysters were alive at trial end (Trial 2) and were negative on histopathological examination for *Bonamia* sp. but had qPCR C_T values of 24.7 and 28.0. The one oyster from Farm 3 that died during the trial period (Trial 2) was *Bonamia*-negative on both qPCR and histopathological examination and was in a different tank to the two *Bonamia*-positive Farm 3 oysters.

Risk factors for oyster death

At the end of the 8-week Tank Trial 1 period, 86 of 250 study oysters (excluding the 10 negative control oysters) had died (34.4%) (Table 11), with significant variation in mortality levels across the different combinations of risk factors.

Table 11. Number of oysters that were dead or alive at the end Tank Trial 1 (56 days), by risk factors (temperature, nutrition and motion)

Risk factors			Status			
Temperature	**Nutrition**	**Motion**	**Dead**	**Alive**	**TOTAL**	**% mort**
Hot	**Fed**	**Tumbled**	10	20	30	33.3
		Still	11	19	30	36.7
	Starved	**Tumbled**	17	13	30	56.7
		Still	11	19	30	36.7
Cold	**Fed**	**Tumbled**	8	22	30	26.7
		Still	6	34	40*	15.0
	Starved	**Tumbled**	7	23	30	23.3
		Still	16	14	30	53.3
		TOTAL	86	164	250	34.4

* includes "positive control" tank (i.e. 4 tanks experienced these conditions)

The effect of the potential risk factors of increased temperature, inadequate nutrition and increased motion on oyster death appeared to be complex, with varying effects depending on the combination of risk factors evaluated (Table 12). For example, starvation alone (i.e. in combination with cold and still water) resulted in a 47% increased risk of death (p<0.01). However, when starvation was combined with a high temperature or increased motion, this caused a significantly decreased risk of death (p=0.03 and 0.02, respectively) (i.e. the effect was reversed). Of all combinations of risk factors, an oyster was at the greatest risk of death when it was held at high temperature, with increased motion and was starved (OR = 1.92; p= 0.01). Twenty-one percent of the variation in oyster status (dead/alive) at the end of the trial was at the tank-level, indicating a proportion of the deaths was associated with within-individual tank effects, as well as stress induced by the risk factors.

Table 12. Results of a generalised linear mixed model evaluating associations between potential risk factors and oyster status (dead/alive) at the end of Tank Trial 1.

Risk factor	β	SE(β)	p-value	OR	LCI	UCI
Starved	0.38	0.11	<0.01*	1.47	1.25	1.69
Hot temperature ("Hot")	0.22	0.11	0.07	1.24	1.02	1.46
Tumbling motion ("Tumbled")	0.12	0.11	0.31	1.12	0.90	1.34
Starved * Hot	-0.38	0.16	0.03*	0.68	0.36	1.00
Starved * Tumbled	-0.42	0.16	0.02*	0.66	0.34	0.98
Hot * Tumbled	-0.15	0.16	0.37	0.86	0.54	1.18
Starved * Hot * Tumbled	0.65	0.24	0.01*	1.92	1.45	2.38

* p<0.05 (statistically significant)
OR = Odds ratio
LCI = Lower Confidence Interval (95%)
UCI = Upper Confidence Interval (95%)
β = Co-efficient
SE(β) = Standard error of the co-efficient

Calculation of diagnostic test sensitivity/specificity and optimal qPCR C_T value cut-point

Histopathological examination applied as gold standard

Histopathological examination was applied as the presumed 'gold standard' test for the diagnosis of bonamiosis in Native Oysters. Oysters that had an 'occasional' or greater number of *Bonamia* sp. in one or more tissues (i.e. 'histopathology-positive') had a significantly lower mean qPCR C_T value relative to histopathology-negative oysters (Figure 16).

The Receiver Operating Curve (ROC) analysis found an AUC for qPCR of 0.92 relative to histopathological examination, indicating a high level of agreement between the two diagnostic tests (Figure 17).

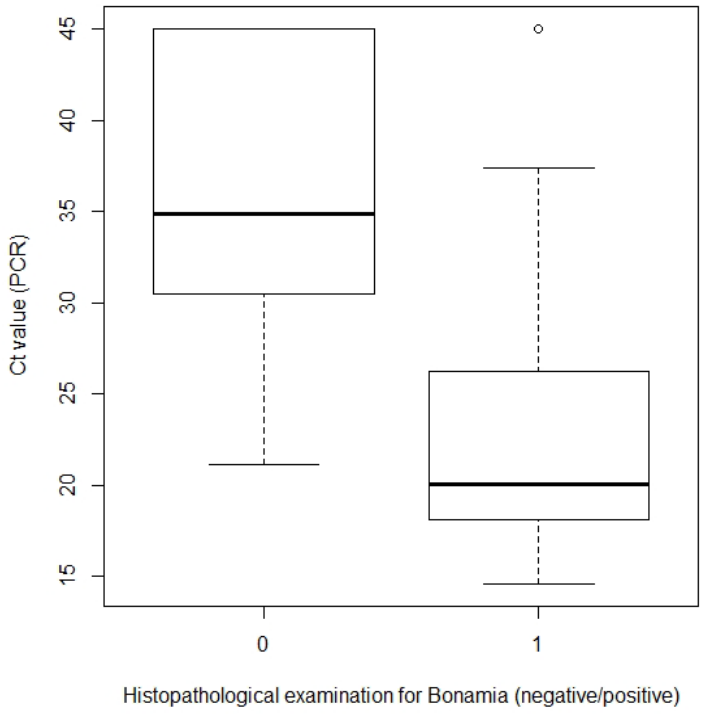

Figure 16. Mean C_T value (neat) (*Bonamia*-specific qPCR) detected in oysters that had 'occasional' or greater *Bonamia* organisms observed (status = 1; n = 107) or not observed (status = 0; n = 125) on histopathological examination at the end of the Tank Trial 2 period (Note: all "undetected" values were re-categorised as 45; the maximum CT value) ($p < 0.01$)

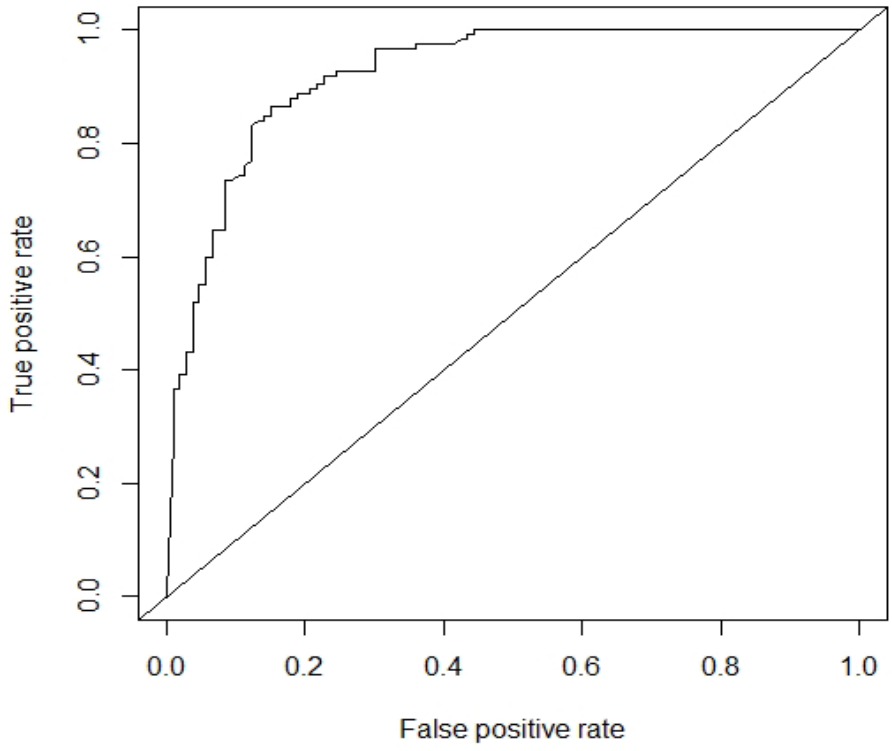

Figure 17. Receiver operating curve (ROC) demonstrating the false positive versus true positive rate for qPCR results when applying histopathological examination as the 'gold standard' test.

Based on this methodology, the optimal qPCR C_T value was calculated at 28.31, with a qPCR diagnostic sensitivity of 85.7% and specificity of 84.9%. This assumed that the cost of false-positive and false-negative errors was equal. However, there was a plateau of approximately 83-85% accuracy between cycle threshold values of 23 and 30, indicating the presumptive cycle threshold of 25 used in the Milestone 4 report for Tank Trial 1 was appropriate (Figure 18).

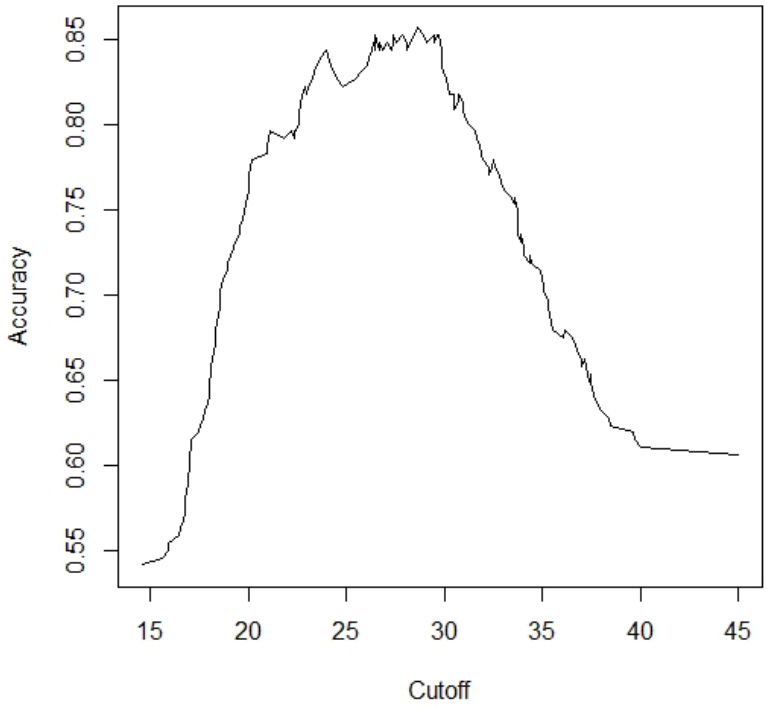

Figure 18. Accuracy of the diagnosis of *Bonamia* sp. in farmed native oysters when applying increasing cycle threshold values for qPCR C_T values, using histopathological examination as the "gold standard" test.

Latent class analyses

The diagnostic Se and Sp of the qPCR were calculated for increasing C_T values from ≤25 to ≤40, with an optimal cycle threshold identified at ≤34.5 (Sp: 92.2; 95% PCI: 76.2, 99.8; and Se: 93.5; 95% PCI: 84.7, 99.1) (Table 13 and Figure 19).

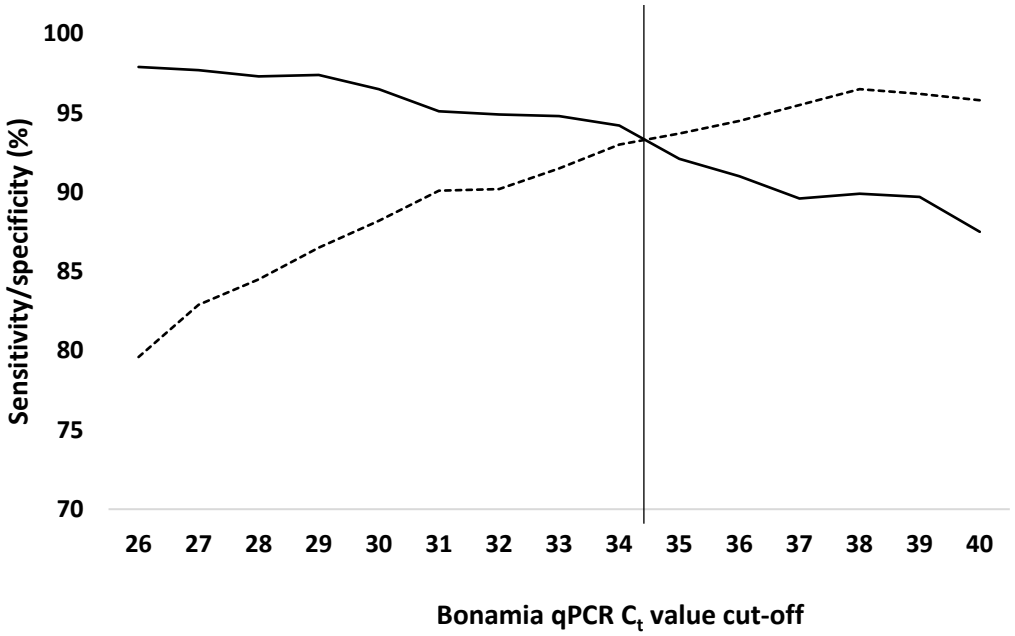

Figure 19. Two-graph receiver operating characteristic plot (sensitivity-specificity plot) with sensitivity (dashed) and specificity (black) calculated for increased threshold cycle (C_T) values for the *Bonamia* sp. real-time polymerase reaction (qPCR) test. Vertical line = sensitivity and specificity equivalent.

Table 13. Cross-tabulation of test results for *Bonamia sp.* in farmed Native Oysters (n=232) in Victoria, Australia, using qPCR and histopathological examination, by qPCR C_T value, and posterior median qPCR sensitivity and specificity estimates (95% posterior credible intervals; PCI).

	Farm 1		Farm 2		Sp	95% PCI	Se	95% PCI
	qPCR C_T cycle threshold value: ≤ 25							
Histo	**+ve**	**-ve**	**+ve**	**-ve**				
+ve	26	22	50	9				
-ve	4	71	5	45	97.9	[91.1, 99.9]	78.5	[70.9, 85.9]
	qPCR C_T cycle threshold value: ≤ 30							
Histo	**+ve**	**-ve**	**+ve**	**-ve**				
+ve	37	11	57	2				
-ve	15	60	12	38	96.5	[86.5, 99.9]	88.2	[80.5, 95.1]
	qPCR C_T cycle threshold value: ≤ 35							
Histo	**+ve**	**-ve**	**+ve**	**-ve**				
+ve	44	4	59	0				
-ve	37	38	27	23	92.1	[76.1, 99.6]	93.7	[84.9, 99.2]
	qPCR C_T cycle threshold value: ≤ 40							
Histo	**+ve**	**-ve**	**+ve**	**-ve**				
+ve	47	1	59	0				
-ve	52	23	38	12	87.5	[68.4, 99.3]	95.8	[84.9, 99.8]

The estimated test characteristics of histopathological examination and the qPCR (at optimal epidemiological cycle threshold of ≤34.5) are detailed in Table 14. The sensitivity of qPCR was significantly higher compared to histopathological examination, while the specificity of both tests was very high (>92.0%). The posterior estimates of prevalence in Farm 1 and 2 were 37.0% (95% PCI: 26.4%-49.3%) and 55.0% (95% PCI: 44.1%-67.2%), respectively.

Table 14. Posterior median and 95% posterior credible intervals (PCI) of the sensitivity and specificity of quantitative polymerase chain reaction (qPCR) (cycle threshold ≤ 34.5) and histopathological examination (Histo) in the diagnosis of Bonamia sp. in farmed Native Oysters, and the estimated prevalence in two independent farms

Parameter	Test/Farm	Estimate	95% PCI
Specificity	qPCR	92.1	[76.1, 99.6]
	Histo	98.7	[94.3, 99.9]
Sensitivity	qPCR	93.7	[84.9, 99.2]
	Histo	50.8	[41.4, 62.3]
Prevalence	Farm 1	37.0	[26.4, 49.3]
	Farm 2	55.0	[44.1, 67.2]

Risk factors for clinical bonamiosis

At the end of the Tank Trial 1 period, 118 live oysters (72.3%) had a qPCR C_T value of >34.5 (i.e. were non-infected). Eighty-six of 240 Farm 1 oysters had died (35.8%) and, of the 63 dead oysters that had tissues available for testing, 17 (27.0%) had a qPCR C_T value of >34.5, indicating death due to a reason other than *Bonamia* sp. infection. Forty-seven oysters had died with clinical bonamiosis at trial end and 45 oysters were diagnosed with subclinical bonamiosis (Table 15).

Table 15. Number of farmed *Bonamia*-positive Native Oysters (n=227) (qPCR C_T value of ≤34.5) after a trial period of 56 days (Trial One) and case fatality rate, by risk factors of interest

Risk factors				*Bonamia*-positive		Case
Water temperature	Nutrition	Water motion	TOTAL*	Number (%) oysters	Number deaths	fatality rate
Hot	**Adequate**	**Tumbled**	24	5 (20.8)	2	0.40
		Still	27	11 (40.7)	9	0.82
	Starved	**Tumbled**	28	22 (78.6)	12	0.55
		Still	28	18 (64.3)	7	0.39
Cold	**Adequate**	**Tumbled**	30	12 (40.0)	8	0.67
		Still	39	11 (28.2)	4	0.36
	Starved	**Tumbled**	28	10 (35.7)	3	0.30
		Still	23	3 (13.0)	2	0.67
		TOTAL	**227**	**92 (40.5)**	**47**	**0.51**

* with tissue available for qPCR testing

There was a 58% increased risk (95% CI: 16%, 99%) of a *Bonamia*-infected oyster dying if the oyster was held at a higher temperature (p=0.05) (Table 16). Although starving and tumbling oysters in isolation was not significantly associated with clinical bonamiosis, a *Bonamia*-infected oyster was at the greatest risk of death when an increased water temperature was combined with both starvation and increased motion (p=0.02; OR=3.47). Twenty-four percent of the variation in oyster status (clinical or subclinical bonamiosis) at the end of the trial was at the tank-level, indicating a proportion of the deaths of *Bonamia*-infected oysters was associated with within-individual tank effects, as well as stress induced by the risk factors.

Table 16. Results of a generalised linear mixed model (GLM) evaluating the significance of associations between potential risk factors and Native Oyster status (positive or negative for *B. exitiosa* infection) after 56 days (Tank Trial 1)

Risk factor	β	SE(β)	p-value	OR	LCI	UCI
Starved	0.30	0.32	0.36	1.35	0.72	1.99
Hot temperature ("Hot")	0.45	0.21	0.05[#]	1.58	1.16	1.99
Tumbling motion ("Tumbled")	0.30	0.21	0.16	1.35	0.95	1.76
Starved * Hot * Tumbled	1.24	0.49	0.02[#]	3.47	2.50	4.44

[#] *p* = statistically significant
OR = Odds ratio
LCI = Lower Confidence Interval (95%)
UCI = Upper Confidence Interval (95%)
β = Co-efficient
SE(β) = Standard error of the co-efficient

Of 240 oysters held in 24 study tanks in Tank Trial 2, four small oysters in three individual tanks did not have tissue available at the end of the study for qPCR and histopathological examination. An additional four oysters did not have tissue available for histopathological examination but were tested using qPCR. At the end of the trial period, 65 live oysters (41.9%) had a qPCR C_T value of >34.5 (i.e. were non-infected). Eighty-three of 240 oysters had died (34.6%) and, of the 77 dead oysters that had tissues available for testing, 2 (2.5%) had a qPCR C_T value of >34.5, indicating death due to a reason other than *Bonamia* sp. infection. Seventy-five oysters had died with clinical bonamiosis at trial end and 90 oysters were diagnosed with subclinical bonamiosis (Table 17).

Table 17. Number of Native Oysters (n=232) sourced from Victoria , *Bonamia*-positive (qPCR C_T value of ≤ 34.5) after a trial period of 83 days (Trial 2) and case fatality rate, by risk factors of interest

Risk factors				*Bonamia*-positive		Case fatality rate
Site	Stressed	Oyster size	TOTAL*	Number (%) oysters	Number deaths	
Farm 1	**No**	**Small**	28	12 (42.9)	0	0.00
		Large	30	14 (46.7)	1	0.07
	Yes	**Small**	35	29 (82.8)	13	0.45
		Large	30	25 (83.3)	6	0.24
Farm 2	**No**	**Small**	29	20 (69.0)	12	0.60
		Large	30	16 (53.3)	9	0.56
	Yes	**Small**	20	20 (100.0)	15	0.75
		Large	30	29 (96.7)	19	0.66
		TOTAL	**232**	**165 (71.1)**	**75**	**0.46**

The mean case fatality rate (i.e. ratio of dead to *Bonamia*-positive oysters) within the study period was higher in oysters sourced from Farm 2 (0.65) than oysters sourced from Farm 1 (0.25) (Table 17). Similarly, the farm from which *Bonamia*--positive oysters were sourced was significantly associated with likelihood of death over the trial period, with an 82% higher risk in Farm 2 oysters versus Farm 1 (Table 18). However, whether the oyster was stressed, when considered in isolation, was not significantly associated with the risk of a *Bonamia*-positive oyster dying (p=0.21). Although smaller oysters had higher case fatality rates and proportion *of Bonamia*-positive individuals relative to larger oysters, where the source and environment were the same (with the exception of unstressed oysters from Farm 1), this relationship was non-significant (p=0.79) (Table 18). There were also no significant interactions between assessed risk factors (p≥0.44). Approximately 17% of variation in the outcome was associated with the tank in which the oyster was located.

Table 18. Results of a generalised linear mixed model (GLM) evaluating associations between potential risk factors and oyster status (subclinical or clinical bonamiosis) at the end of the Trial 2 (83 days)

Risk factor	β	SE(β)	p-value	OR	LCI	UCI
Farm	0.60	0.23	0.02[#]	1.82	1.36	2.27
Stressed	0.65	0.49	0.21	1.92	0.95	2.89
Oyster size	0.14	0.53	0.79	1.15	0.11	2.19
Stressed * Farm	-0.25	0.32	0.44	0.78	0.16	1.40
Stressed * Oyster size	-0.38	0.69	0.59	0.68	-0.68	2.04
Farm * Oyster size	-0.08	0.33	0.80	0.92	0.28	1.56
Stressed * Farm * Oyster size	0.16	0.44	0.72	1.17	0.31	2.04

[#] p = statistically significant
OR = Odds ratio
LCI = Lower Confidence Interval (95%)
UCI = Upper Confidence Interval (95%)
β = Co-efficient
SE(β) = Standard error of the co-efficient

Survival analysis – risk factors for time to oyster death

Oysters died at a consistent rate in both trials (Figure 20). The probability that a *Bonamia*-infected oyster would survive beyond a specified time (i.e. the estimated survival function) up to 56 days (Tank Trial 1) was not significantly associated with an increased water temperature, starvation or agitation (p>0.05). However, in Tank Trial 2, when the estimated survival function was stratified by source farm (1 or 2) and application of stress (stressed/non-stressed), the death of *Bonamia*-infected oysters was influenced by both risk factors. Although, prior to the trial start date, there was a lower mortality rate in Farm 2 oysters in the field, during Tank Trial 2, *Bonamia*-infected oysters sourced from Farm 2 died at 3.76 times the rate per unit time as *Bonamia*-infected Farm 1 oysters (Figure 20; Table 19). The association between clinical bonamiosis and stress was more complex, with a higher HR recorded in stressed Farm 1 oysters, but the opposite effect in Farm 2 oysters, particularly during the first 40-50 days of the trial period (Figure 20). Oyster size did not have a significant influence on the HR (p=0.35).

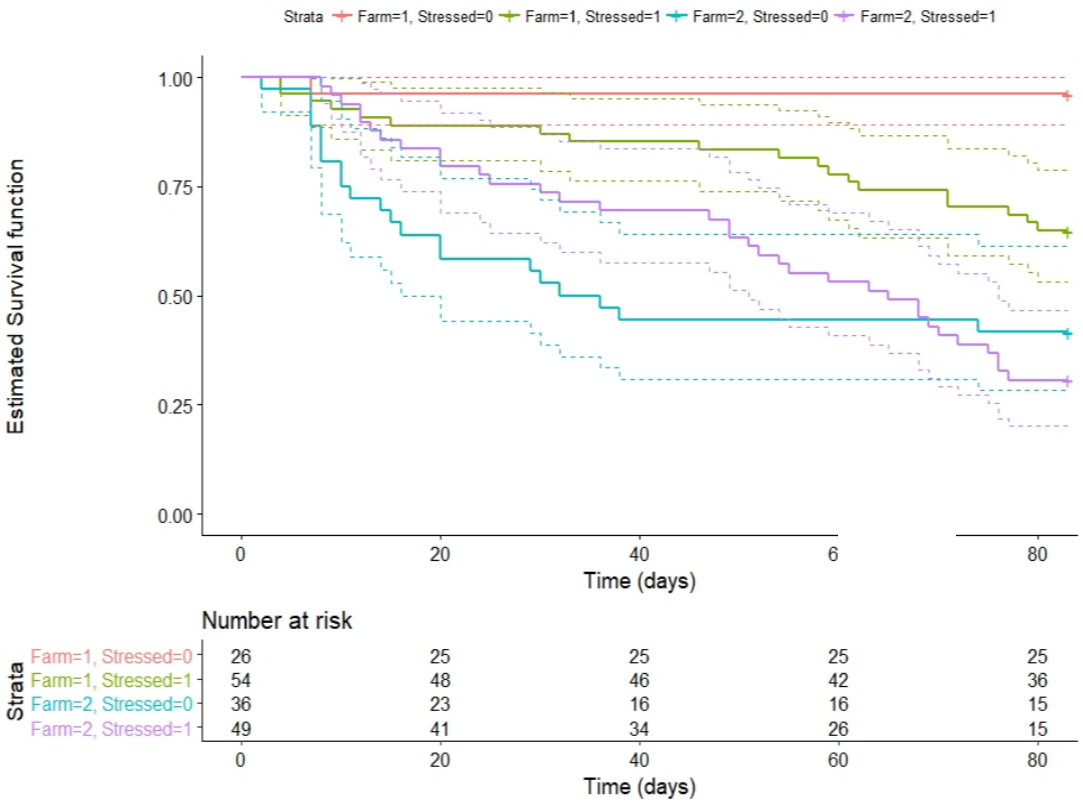

Figure 20. Risk table and Kaplan-Meier plot with 95% confidence bounds of survival of *Bonamia* positive farmed Native Oysters from Victoria, Australia, stratified by source Farm and application of stress (Farm 1/non-stressed (n=26): orange; Farm 1/stressed (n=54): green; Farm 2/non-stressed (n=36): blue; Farm 2/stressed (n=49); purple), over the trial period (Tank Trial 2: 83 days)

Table 19. Estimated (β) from the Cox model of the hazard ratios (HR) of potential risk factors on the risk of death in *Bonamia*-positive farmed Native Oysters from Victoria, Australia (n=165 oysters).

Risk factor	β	Robust SE(β)	p-value	HR	LCI	UCI
Farm	1.32	0.47	<0.01[#]	3.76	1.49	9.49
Stressed	0.43	0.31	0.17	1.53	0.84	2.81
Oyster size	-0.31	0.34	0.35	0.73	0.38	1.41

[#] p = statistically significant
HR = Hazard ratio
LCI = Lower Confidence Interval (95%)
UCI = Upper Confidence Interval (95%)
β = Co-efficient
SE(β) = Standard error of the co-efficient

4.4 Discussion

Histopathological analysis

In line with previous work by Corbeil (Corbeil et al., 2009), *B. exitiosa* was observed most commonly and in the highest numbers within the interstitium, digestive gland and palp and least commonly in the stomach. The interstitium and/or the digestive gland as a tissue type yielded 80% of the diagnoses of *Bonamia*-positive oysters. Contrary to the observations of Corbeil et al. (2009), oysters that died during the trial appeared to be systemically infected with *Bonamia* sp. as organisms were observed in four or more tissues within 83% of infected, dead oysters. This indicates that dissemination of the parasite throughout an oyster was associated with death. It is likely that in the wild, a heavily infected, moribund oyster is either rapidly predated or tissue disintegration prevents the ability to recover tissue that could illustrate the systemic nature of *Bonamia* sp. infection. During this trial, with very frequent assessment of oyster status and the absence of predators we were able to demonstrate the systemic nature of infection in heavily infected oysters (Hine et al., 2002).

Tank Trial 1

Tank Trial 1 examined the effect of a range of treatments on oyster survival. The relationship between the risk of an oyster dying under the effect of a single or combined risk factor was not straightforward. Where starvation was combined with cold and still water (i.e. no other risk factors applied), the 47% increase in risk of death is biologically plausible. However, the protective nature of starvation and elevated water temperature (warm), for example, is more difficult to explain. In combining all three risk factors: starvation, high temperature and increased motion (tumbling); there was a significant increase in the risk of death (OR = 1.92, p=0.01). The selection of risk factors was determined by consultation with farmers and previous results in the literature. As for many other disease processes, it is considered plausible that increased stress will result in an increased susceptibility of oysters to *Bonamia* sp. infection. Anecdotally, overcrowding of baskets, poor feed availability and other water quality issues have been given as causes for outbreaks of bonamiosis in Port Phillip Bay.

Applying a range of potential stressors to oysters, Hine et al. (2002) found that hyposalinity, and exposing oysters to the air for eight hours resulted in the highest mortality rates. In the same experiment it was found that the treatments that resulted in the highest overall prevalence of *Bonamia exitiosa* (measured by histopathology) in both dead and surviving oysters was hot water, exposure to air and stir treatment (Hine et al., 2002). Although this work was undertaken in New Zealand with *Ostrea chilensis* under different conditions, the results are considered to be comparable.

The outcome of qPCR tests, such as that used to diagnose *B. exitiosa* infection in Native Oysters, is continuous and the distribution of C_T values are generally non-normal (Burns & Valdivia, 2008). A cycle threshold value, above which a test would be

considered false, is defined based on utilising either an analytical or epidemiological approach (Caraguel et al., 2011). An analytical approach develops a C_T value based on criteria gathered during assay development (i.e. within the laboratory) and, within Tank Trial 1, an analytical qPCR C_T was applied to differentiate positive from negative samples.

In contrast, an epidemiological C_T value is developed based on the probability of a false test result. Calculation of an epidemiological C_T, versus use of an analytical C_T, has been increasingly applied in recent years to reduce the probability of and costs associated with misclassification, most typically related to terrestrial animal diseases (Vandenbussche et al., 2008, Mahmmod et al., 2013, Nielsen et al., 2015). The outcome in Tank Trial 2 was informed by the calculation of the optimal epidemiological qPCR C_T value to differentiate between a positive and negative result. The development of this optimal C_T is dealt with in the section below.

Calculation of diagnostic test sensitivity/specificity and optimal qPCR cycle threshold value (C_T)

The comparison of PCR C_T value and histopathology (i.e. *Bonamia* sp. present or absent) yielded a high level of agreement (0.92) with an "Area Under Curve" in the ROC analysis. Histopathology has traditionally been considered the "gold standard" for detection of *Bonamia* sp.*,* however this diagnostic technique cannot discern past the genus level (Diggles et al., 2003).

Histopathology is recommended as the surveillance method of choice in regions infected by *B. exitiosa* with PCR recommended as a confirmatory test only (OIE, 2018). When examining the diagnostic sensitivity and specificity of molecular tests for *B. exitiosa* there are a range of values provided in the literature. The PCR for *Bonamia* sp. in *O. chilensis* has been previously validated with a sensitivity of 88% and a specificity of 36% relative to a "gold standard" combination of histology and heart imprints (Diggles et al., 2003). More recently, comparing histology/smear and qPCR, (Buss et al., 2018) found the diagnostic sensitivity of histology to be greater than qPCR (76% versus 69%) with a specificity equivalent to 93%. Given the progression of the pathologic process from initial infection with the parasite to recognisable tissue changes, it is reasonable to conclude that molecular detection of infection is likely to be more sensitive than conventional histopathology as shown by others (Ramilo et al., 2013) and in this study.

The qPCR used in this paper was developed by our collaborators at CSIRO and had not until this project been formally validated. The results from this project suggest from the latent class model across two farms, each with a different infection prevalence, that the *Bonamia* sp. qPCR yielded a relatively high diagnostic sensitivity of 93.7% when compared with histopathology at 50.8%. The specificity of both tests was very high at over 92.1%. The optimal C_T for this assay as an indicator of infection with *Bonamia* sp. was determined to be ≤34.5.

Risk factors for clinical bonamiosis Tank Trial 1:

Following establishment of the optimal epidemiological C_T to determine positive and negative bonamiosis status using the CSIRO qPCR, the risk factors associated with clinical bonamiosis in *Bonamia* - positive oysters were determined. Surprisingly, starvation alone was not a significant risk factor, however, as was the case for risk factors related to oyster death across the whole study population (described earlier), supplying the oysters with warm water alone increased the risk by 58% of a *Bonamia* - positive oyster dying. The odds of a *Bonamia* - positive oyster dying when all three stressors were applied (heat, starvation and tumbling) were almost three and a half times greater than for a positive oyster not subjected to those stressors. The magnitude of this result was greater than when examining the effect of these factors on all (positive and negative oysters).

Early observations on the oyster *Ostrea edulis* in the Netherlands (Banning, 1991) concluded that the prevalence and development of bonamiosis related to stress and environmental factors. Hine (2002) found temperature had the greatest influence on overall prevalence of *Bonamia* sp. in both dead and surviving oysters subjected to a range of treatments. The mean ambient water temperature during Tank Trial 1 was 18.2°C and the mean heated temperature was 22.9°C. Temperatures recorded by loggers on the site in Port Phillip Bay have reached over 23°C but only for a few days at a time, so it is most likely that the sustained elevation in temperature during the tank trial was detrimental to the health of oysters. Port Phillip Bay is considered shallow with an average depth of 12 metres. Historical data collected by CSIRO at a comparable site in the late 1960s (King, 1970) determined that the highest monthly average at the time was 20.6°C. Despite different instrumentation, the monthly mean water temperatures during this study was over 2 degrees higher. It seems highly likely that as marine waters increase in temperature the detrimental effects of pathogens such as *Bonamia* sp. will be increased.

Risk factors for clinical bonamiosis Tank Trial 2:

Tank Trial 2 was designed to examine the findings from Field Trial 1. Specifically, that smaller oysters had a higher mortality rate than larger oysters, as well as evaluating the effects of stressors (heat and starvation) on the development of clinical bonamiosis from both the clinically infected farm (Farm 1) and subclinically infected farm (Farm 2). Using the newly established epidemiological qPCR C_T, it was confirmed that by the end of the trial 75 oysters were found to have died with clinical bonamiosis and 90 oysters were diagnosed with subclinical bonamiosis. Only 2 oysters died with a C_T value of >34.5 from which it was inferred they died from a cause other than infection with *Bonamia* sp. From the variables applied, the only significant factor that emerged from the GLM was the source farm, Farm 2 had an 82% higher risk of death during the trial period. This result was replicated in the mean case fatality rate. The survival analysis also demonstrated a significant association between death and oyster provenance. The estimated hazards ratio for *Bonamia* sp. infected oysters sourced from Farm 2 was 3.76 indicating that these oysters died at 3.76 times the rate per unit time than those sourced from Farm 1.

Interestingly, the daily hazard of death was significantly associated with farm, but the effect of stress differed between the farms. As expected, the mortality rate was greatest in Farm 1 oysters that were stressed. However, a similar effect was not replicated on Farm 2. Although smaller oysters were significantly associated with mortality on Farm 1 during the field trial, this effect could not be replicated during Tank Trial 2. This indicates that, although smaller oysters were more likely to die on Farm 1, this may not have been due to clinical bonamiosis.

The molluscan immune system relies on an innate cellular response and lacks the humoral response characterised by antibodies and lymphocytes (Morga et al., 2012). For this reason, vaccines cannot be deployed in response to molluscan diseases. The main mechanisms for mitigating and excluding pathogens are by implementing biosecurity controls and the development of resistant molluscs (Morga et al., 2012). In foundation work on the protozoan parasite *Perkinsus marinus* in *Crassostrea virginica,* it was concluded that populations exposed to the parasite for more than 40 years developed resistance (Bushek & Allen, 1996). More recently, Dégremont et al. (2015) demonstrated heritability in *C. gigas* spat to the virus ostreid herpesvirus 1 with strong gains recorded in selected spat over the control in 3 years.

The major finding for the GLM Tank Trial 2 was that oysters from Farm 2 were at a much higher risk of dying from clinical bonamiosis during the trial than oysters from Farm 1. Geographically, Farms 1 and 2 are located in different bays, approximately 70km apart and separated by a 10km wide land peninsula. The stock at Farm 1 were derived from farmed brood stock within Port Phillip Bay which were spawned at a local hatchery. The stock at Farm 2 came from wild brood stock from Westernport Bay that were spawned locally. Both sites have had a history of bonamiosis, however Farm 2 has not recorded clinical bonamiosis since stock re-establishment in recent years. Following the major clinical outbreak of bonamiosis on Farm 1 in 2015 it was assumed that up to 80% of the original stock was destroyed by the parasite. No extensive surveys to determine prevalence had been undertaken at either site however. The age classes of stock on both farms is uncertain, but it is possible that some of the oysters at either farm may have been as old as 5 years. One hypothesis is that the stock from Farms 1 and 2 have quite distinct genetic profiles and that more recent selection pressures have resulted in survivors of Farm 1 oyster stock being less susceptible to developing clinical bonamiosis.

Field Trials 1 and 2

The field trials undertaken in this project aimed to investigate the commonly held beliefs by Victorian flat oyster farmers relating to the occurrence of clinical bonamiosis on their farms. As outlined in Appendix 1, all the Victorian farms utilise submerged basket (or rope) culture. As such, the stock are not examined regularly and the ability to recover tissue from a dying oyster to establish cause of death is virtually impossible. The surrogate measure for bonamiosis therefore was oyster mortality which is clearly a non-specific measure. Field Trial 1 examined the risk factors of basket density,

oyster size and depth of the basket in the water. The results from this trial were complex but it did appear that there were a range of mortality rates with some factors appearing more protective (for example large oysters at 30% stocking and 2 metres depth) and some factors potentially imposing greater stress on the control oysters (small oysters, 60% stocking and 2 metres depth). The one factor that was significantly associated with a higher mortality rate was oyster size; smaller oysters have a higher mortality rate than larger individuals.

It is biologically plausible, and has been observed in some studies, that older (and presumably larger) oysters are more likely to be infected with *Bonamia* sp. In examining the prevalence of *B. ostreae* in *Ostrea edulis* in Ireland, two years of age was found to be the critical age for developing bonamiosis (Culloty & Mulcahy, 1996). However, in this Victorian study, there was limited information about the year classes of stock. It is likely, particularly in the second year of the study, that the smaller oysters were not younger but in fact stock that failed to thrive for unknown reasons.

Intuitively, the level of crowding and fouling in a basket should affect the stress level of the oysters, however, this was not found to be the case in this study. It is possible that there were other major causes of disease operating in this trial, such as the highly prevalent and destructive “mud worm” endemic to these waters. The inability to confirm cause of death in this study was clearly a major limitation. However, in future studies it would be valuable to establish a more frequent regime of stock examination.

4.5 Conclusion

This project has been successful in accomplishing its planned objectives as well as some additional subsidiary ones:

- There was a significant increase in the risk of oyster death during the tank trial when three proposed risk factors were combined: starvation, high temperature and increased motion (tumbling).

- The odds of a *Bonamia* - positive oyster dying during the tank trial when all three stressors were applied (heat, starvation and tumbling) were almost three and a half times greater than for a positive oyster not subjected to those stressors. Further, supplying the oysters with warm water alone increased the risk by 58%of a *Bonamia* - positive oyster dying

- From two separate forms of analysis in the second tank trial, oyster origin was found to be a significant factor such that the farm that had previously not experienced clinical bonamiosis died at almost 4 times the rate of oysters from the farm that had experienced clinical bonamiosis.

- The CSIRO qPCR yielded a relatively high diagnostic sensitivity of 93.7% when compared with histopathology at 50.8%. The specificity of both tests was very high at over 92.1%. The optimal C_T for the assay was determined to be a value of ≤34.5 (as an indicator of infection with *Bonamia* sp.).

- The field trials over two years yielded a range of results. In the first year, smaller oysters appeared to suffer higher levels of mortality (aetiology unconfirmed) but in the second year there was no apparent pattern of mortalities. In both years the levels of mortality were unsustainably high.

4.6 Appendices

APPENDIX A: Descriptive findings from field trips to affected farms prior to Victorian Project commencement.

Farm 1 – first infected farm, Grassy Point site

Farm 1 primarily used basket culture and had between 50,000 and 100,000 oysters in the water prior to the project start date. Precise details about stock on the farm were unavailable at that time following a partnership breakup with a subsequent loss of management knowledge. The baskets were stocked 33% to 66% in volume and the grow-out baskets were generally 650 mm long with 20 mm mesh. In 2013, the farm had 2,000 large baskets (1,100mm) and 6,000 x 650mm baskets. Baskets were previously de-fouled every month, but at the time of initial sampling (March 2015) it appeared that this activity hadn't been undertaken for some months. A smaller (unknown) proportion of stock were being grown glued to droppers at 5 cm to 10 cm distance between oysters. Stock were grown in water depths of 3m to 8m (total depth in this area is 11m). Ages of stock seeded on this farm (in March 2015) were 2012, 2013 and 2014.

During sampling in March 2015, it appeared that there was approximately 70% mortality on Farm 1. The stock, although in good condition, were overgrown with fouling organisms and mussels.

Subsequent sampling was undertaken in June 2015. There was little information that could be gleaned from the stock on the farm so measurement of size (as a surrogate measure for age) was undertaken. There is a common belief that older (and therefore generally larger) oysters are more likely to succumb to bonamiosis (Culloty & Mulcahy, 1996). To examine this proposition a selection of oysters was sorted and measured. Oysters kept in basket culture (three baskets sampled) had an overall mortality of 12.7% from 110 sampled. The mean size of these 110 oysters was 6.16 cm (+/- 0.14 cm). The size of the live oysters was 5.93 cm (+/- 0.12 cm) and the size of the dead oysters was 7.68 cm (+/- 0.62 cm). The seeding of these oysters was unknown, and they appeared to be a collection of re-sorted animals.

The common belief amongst famers is that oysters in baskets are more likely to be affected by bonamiosis. On Farm 1, a single dropper was opportunistically selected and sampled to assess for mortalities and length of oyster (dead and alive). A total of 95 oysters were sampled and the overall mortality rate was 66.3% (much higher than

the basket oyster mortality rate). The mean size of all oysters was 7.81 cm (+/- 0.11). The dead oysters measured 7.6 cm (+/-0.11) and the live oysters measured 8.30 cm (+/-0.20). There are obviously assumptions made in these observations around the cause of death (not confirmed as bonamiosis) and the generalising of results from a small sample to the whole farm.

In August 2015, another opportunity was taken to observe stock on Farm 1. Farm staff reported sorting 10 sets of 3 baskets of which approximately 90% of the stock were dead. Stock were sorted from baskets in the presence of a departmental staff member. In baskets 1 to 3, as a vertical set, 154 oysters were sampled with 53 alive and 101 dead, yielding a mortality rate of 66%. From baskets 4 to 6 there were 169 oysters sampled with a mortality rate of 61%. There was no opportunity to measure oyster length during this field trip.

Farm 1a – second infected farm, Grassy Point site (this farm was not part of the project).

Farm 1(a) is located approximately 90 metres from Farm 1 and had lost all stock that was held in baskets. The reason for this mortality was not known. There is no prior history of testing at Farm 1(a) and there was no histopathology undertaken during the outbreak on oysters from this farm. This farm had less than 50,000 oysters in the water on long lines and had approximately 5,000 oysters in the water at the time of this report. Dropper 1 was seeded in June 2014 and, of 82 sampled, 25 were dead, yielding a mortality rate of 30.5%. The overall size of oysters was 6.76 cm (+/- 0.11 cm) with the mean size of dead oysters as 6.94 cm (+/-0.20 cm) and live as 6.88 cm (+/-0.13 cm). Dropper 2 was seeded in March 2014 and, of 105 sampled, 48 were dead. The mortality rate was 45.7% with the mean size overall of 7.48 cm (+/-0.13 cm) and of dead oysters 7.20 cm and alive 7.71 cm. No further visits were undertaken at Farm 1(a).

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5. SARDI - Diagnostic performance of qPCR, tissue smears and histology for understanding field prevalence and intensity of *Bonamia exitiosa* in *Ostrea angasi*

5.1 Summary

Bonamia threaten flat oyster (*Ostrea* spp.) farming worldwide. Understanding test performance is important for designing surveillance and interpreting diagnostic results. We tested oysters from three farms for *Bonamia exitiosa* using heart smear, histology and qPCR. We used a Bayesian Latent Class Model to assess diagnostic sensitivity (DSe) and specificity (DSp) of these tests individually or in combination, and to assess prevalence. Histology was the best individual test (DSe 0.76, DSp 0.93) compared to quantitative polymerase chain reaction (qPCR) (DSe 0.69, DSp 0.93) and heart smear (DSe 0.61, DSp 0.60). Histology combined with qPCR and defining a positive from either test as an infected case maximised test performance (DSe 0.91, DSp 0.88). Prevalence was higher at two farms in a high-density oyster growing region than at a farm cultivating oysters at lower density. Parasite intensities were lower than in New Zealand and European studies and this is probably contributed to differences in the performance of test when compared to other studies. Understanding diagnostic test performance in different populations can support development of improved *B. exitiosa* surveillance programs.

5.2 Introduction

Surveys in 1992-93 did not detect *Bonamia* in *O. angasi* in South Australia (SA) (Handlinger et al., 1999). A Pacific oyster health survey in South Australia identified *Bonamia*-like cells in healthy *C. gigas* using histopathology in 2003, but the diagnoses could not be confirmed (Diggles, 2003). This finding is, however, consistent with Lynch et al. (2010) who recorded *Bonamia* in *C. gigas* from Spain and Ireland using polymerase chain reaction (PCR), histology and *in situ* hybridisation (ISH). A pilot survey in SA in 2015-16 (unpublished data, confirmed by the Australian Animal Health Laboratory and reported to the Office International des Epizooties (OIE)) of *O. angasi* using quantitative PCR (qPCR) and histology detected *Bonamia*. These results were difficult to interpret due to between-study differences in testing approaches, along with a limited quantitative understanding of the performance of these tests.

5.3 Objectives

We aimed to better understand diagnostic performance of tests for *B. exitiosa*. We tested *O. angasi* from a hatchery and three farm sites in SA for *B. exitiosa*. A Bayesian

Latent Class Model (LCM) was used to assess DSe and DSp of heart smear, qPCR and histology applied singularly or in combination, allowing for covariance between tests, and disease prevalence. The model was also used to calculate LRs and PVs based on predicted DSe, DSp and prevalence. Our study used SA *Ostrea angasi* farms as a test case to contribute to the development of a rigorous method to assess *B. exitiosa* prevalence or establish freedom from infection.

5.4 Methods

Ostrea angasi were sourced from a hatchery and three farm sites in two oyster farming areas. Farmed samples were obtained from two sites in Coffin Bay (34.6236° S, 135.4655° E) (denoted A and B) and one in Streaky Bay (32.7972° S, 134.2111° E). Hatchery-reared spat were sourced from the South Australian Research and Development Institute (SARDI) SA Aquatic Sciences Centre (SAASC) (West Beach, Adelaide, SA) Mollusc Hatchery.

Ostrea angasi were sampled following a randomisation plan where 15 oysters were collected per basket, with 10 baskets chosen per farm site or hatchery. Only 100 oysters were sampled from Coffin Bay A due to poor weather conditions, while 150 individuals were collected from other sites. Oysters were collected, stored in a cooler box, and transported ashore (when required) for sampling. Oysters were weighed to three decimal places and the longest shell axis (hinge to top of shell) length was measured with digital calipers (Craftright 150 mm, Stainless Steel Digital Vernier Caliper). Oysters were shucked, the oyster tissue was removed, the empty shell weighed and the meat to shell ratio calculated ([meat weight (g) / shell weight (g)] x 100). After shucking and weighing, oyster tissues were processed for diagnostic testing. Three diagnostic tests, listed in the OIE Diagnostic Manual for detection of *B. exitiosa* (OIE, 2017) were applied to each sample: heart smear, qPCR and histology. Oysters from the hatchery were too small for heart smear analysis but were assessed by qPCR and histology.

Table 20. Size data (mean ± standard error), prevalence (calculated by the Latent Class Model (LCM)) and mean intensity (calculated through Quantitative Parasitology) of *Bonamia exitiosa* in *Ostrea angasi* from heart smears, histology and qPCR, June/July 2016, South Australia[†‡]

	Size data				LCM	Quantitative parasitology (bootstrap 95%)	
Site	**Weight (g) (Mean±SE)**	**Shell length (mm) (Mean±SE)**	**Meat:shell ratio (%) (Mean±SE)**	**n**	**LCM prevalence (credible intervals)[§]**	**Mean heart smear intensity cell count (confidence intervals)**	**Mean histology intensity cell count (confidence intervals)**
Coffin Bay A	66.05±1.48	71.34±1.23	32.04±0.71	100	**0.90 (0.78–0.99)[a]** 0.91(0.78–1.00)[a]	4.36 (3.72–5.04)[a]	4.07 (2.88–6.21)[a]
Coffin Bay B	27.00±0.87	59.96±0.59	45.36±0.73	150	**0.90 (0.78–0.99)[a]** 0.85 (0.71–0.98)[ab]	3.85 (3.2–4.67)[a]	1.88 (1.69–2.10)[b]
Streaky Bay	30.59±0.95	58.62±0.75	34.89±0.68	150	**0.59 (0.46–0.72)[b]** 0.59 (0.46–0.73)[b]	3.92 (3.39–4.66)[a]	1.55 (1.35–1.67)[c]
Hatchery	0.62±0.02	16.67±0.01	20.14±0.40	150	N/A	N/A	N/A

† Different superscripts denote differences at a 5% level, with [a] representing the highest value.

‡ SE: standard error; n: sample number per site; N/A: Not applicable.

§ Prevalence values in bold were from Model–1, where indeterminate (one positive and one negative result within duplicate) qPCR samples were deemed as positive and non-bold values were from Model–2, where indeterminate qPCR samples were deemed as negative.

A heart smear was prepared from each oyster by removing the heart from the pericardial cavity with fine forceps. Excess liquid was briefly blotted on filter paper, and the heart was lightly touched on a labelled microscope slide with 10 tissue imprints per slide. Slides were air dried for at least 5 min, dipped in methanol, re-dried and stained using a Hemacolor® kit (Merck). The slides were mounted with a cover slip and DPX (Sigma-Aldrich).

After the heart smear was made, a diagonal 3–5 mm tissue section was taken from each oyster ensuring each sample included mantle, gills, digestive gland and gonad. This section was placed in a histology cassette and fixed in 10% formalin with filtered seawater for 48 h and transferred to 70% ethanol for storage. Samples were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin. Heart smear and histology slides were examined at 400x and 1000x (with oil immersion) with a compound light microscope (Brightfield Olympus BX53 Upright Microscope), the number of *B. exitiosa* cells per slide were counted and scored using a system derived from Diggles, Cochennec-Laureau and Hine (2003) with further description distinguishing very light and light infection grades for histology, as these were most common (Table 21).

Table 21. Grading of infection intensity by *Bonamia exitiosa* for heart smears and histology in *Ostrea angasi* derived from Diggles et al. (2003)

Grading of infection	Heart Smear description	Histology description
0 – Not infected	No *Bonamia* cells present.	No *Bonamia* cells present.
1 – Very light infection	One *Bonamia* cell observed per heart smear.	One *Bonamia* cell observed per slide after extensive searching.
2 – Light infection	Between 2–10 *Bonamia* cells present per heart smear.	Between 2–10 *Bonamia* cells observed after searching, 1–2 cells per infected haemocyte.
3 – Moderate infection	More than 10 *Bonamia* cells per heart smear, but few parasites per haemocyte.	More than 10 *Bonamia* cells per slide, infection widespread and diffuse. Only 1–5 cells per haemocyte.
4 – Heavy infection	*Bonamia* present in many haemocytes per heart smear, and many parasites per haemocyte.	*Bonamia* cells readily observed in one or more tissues, often associated with haemocytosis.
5 – Systemic infection	*Bonamia* present in virtually all haemocytes in each heart smear, many parasites in each haemocyte and extracellularly.	*Bonamia* cells abundant in all tissues, many per haemocyte and often extracellular. Haemocytosis always present and lesions sometimes observed.

A 5 x 5 mm sample of mantle and gill and the tissue remaining from the heart smear were preserved in 70% ethanol. DNA was extracted with the QIAamp Mini kit (#51306, Qiagen), following the manufacturer's protocol from a pooled heart, gill and mantle tissue sample (total mass of approximately 25 mg) from each oyster. A negative extraction control (no tissue) was also included. Extracted DNA quality and

concentration were assessed using a Nanodrop® ND-2000 spectrophotometer (Thermo Fisher Scientific). Extracted DNA was stored at –20°C.

To ensure samples contained amplifiable DNA, extracted samples were assayed using the Taqman® Ribosomal RNA Control Reagents kit (#4308329, Applied Biosystems). Thermal cycling was performed according to manufacturer's guidelines up to 45 cycles using a StepOnePlus (Applied Biosystems). Samples were then tested with the Corbeil et al. (2006) *Bonamia* sp. qPCR assay in 96-well plates in a 25 µL reaction volume using the following chemistry: forward primer (5'-CCC TGC CCT TTG TAC ACA CC 3'); reverse primer (5' TCA CAA AGC TTC TAA GAA CGC G-3'); carboxyfluorescein (FAM)-labelled probe (minor groove binder/non-fluorescent quencher) (5'6FAM- TTA GGT GGA TAA GAG CCG C MGB-3'). The 23 µL master mix contained 12.5 µL Universal Master Mix, 6.75 µL water, 1.25 µL of each primer (18 µM) and TaqMan probe (5 µM). 2 µL aliquots of DNA were added to the master mix and thermal cycling was performed using a StepOnePlus (Applied Biosystems) machine: 50°C for 2 min, followed by 95°C for 10 min and 45 cycles of 95°C for 15 s and 63.6°C for 60 s (Corbeil et al., 2006) with a threshold of 0.1. DNA from all samples were tested in neat and 1:10 dilution duplicates. Each plate included a positive plasmid control (Australian Animal Health Laboratory, East Geelong, Victoria), a no template control and a negative extraction control. For each sample, if one replicate was positive and one undetected, the sample was deemed indeterminate and the sample was re-tested. Any samples with indeterminate results for the 18S Internal-Control were re-tested unless the *Bonamia* sp. assay result was positive. A sample was described as positive when there was statistically significant increase in fluorescence output above the background (Corbeil et al., 2006), meaning the cycle threshold (C_T) was reached and a typical amplification curve was displayed. C_T values were calculated automatically using StepOne™ Software v. 2.3. When a sample did not have a C_T value or a typical amplification curve the sample was described as negative.

DSe and DSp of the three tests and true prevalence for each farm site were estimated using a Bayesian LCM allowing conditional dependence (covariance) between tests, following Lewis and Torgerson (2012). Code is provided in supplementary material to Buss et al. (2018).

Hatchery samples were not included in the LCM because *B. exitiosa* was not detected by histology or qPCR in these 150 samples. True prevalence of *B. exitiosa* in the hatchery was therefore estimated in a Bayesian framework using the R package 'prevalence' (Devleesschauwer et al., 2015). For this analysis beta (4, 3) priors, reflecting 95% confidence that these parameters are between 0.3 and 0.99 with expected mean of 0.6, were used for DSe and DSp. Posterior predictions for DSp from this analysis were used to inform priors for DSp of histology and qPCR in the LCM. For these two parameters, beta (20.1, 0.3) priors were therefore used, reflecting 95% confidence that the true histology and qPCR DSp values are between 0.90 and 1 with expected mean of 0.98. Where strong prior knowledge was not available for the DSp of heart smear and all DSe parameters, weakly informative beta (4, 3) priors were applied. Uninformative beta (1, 1) priors were applied to prevalence from each of the three farm sites to negate any prevalence site assumptions. Normal priors with mean

0 and precision 9 were used for all covariance terms to allow for either positive or negative covariance with 95% confidence that covariances are <|0.65|.

Two models using the same priors but with different classifications for qPCR results that remained classed as indeterminate after retesting were run: Model–1 defined indeterminate cases for qPCR as positive and Model–2 defined indeterminate cases for qPCR as negative.

DSe and DSp of all two-test combinations (based on Weinstein et al., 2005), LRs (Caraguel & Vanderstichel, 2013) and PVs (Fegan, 2000) of all individual and combined tests, were generated from deterministic nodes within the model, using DSe and DSp from single or combined tests as appropriate for LR and PV calculations. Note that all diagnostic tests were performed concurrently for each specimen without regard to results of other tests, i.e. in parallel. Between-test correlations were calculated from deterministic nodes based on predicted covariances and DSe or DSp as appropriate of the relevant tests. Markov Chain Monte Carlo (MCMC) simulations were obtained by running the model in JAGS v. 4.3.0 (Plummer, 2017) using three chains for 50,000 iterations, thinned at a rate of 50, following 2,000 iterations for adaptation and 10,000 iterations for burn-in. JAGS was run using the 'R2jags' package (Su & Yajima, 2015) in R (R Core Team, 2017). Convergence was assessed by Gelman-Rubin convergence statistic, and confirmed by visual inspection of trace, density and autocorrelation plots generated using the 'MCMCvis' package (Youngflesh, 2018).

Parameter values were compared based on their posterior probability distributions, and we report the mean and 95% credible intervals for each based on 3,000 simulations. Covariances between tests were considered important where their 95% credible intervals (CI) did not cross zero.

Bonamia exitiosa intensity cell counts from histology and heart smears were analysed per farm site using Quantitative Parasitology v. 3.0 (Reiczigel & Rózsa, 2005). Mean intensity 95% bootstrap confidence intervals with 2000 replicates were used to assess differences in intensity between farms, with overlapping intervals signifying no difference. In addition, generalised linear models (GLM) were used to identify the relationship between *B. exitiosa* intensity and site, oyster weight (g) and meat:shell ratio (%), for both the heart smear and histology methods. We assumed a negative binomial distribution for intensity data which were over-dispersed relative to a Poisson distribution. GLMs were conducted in R using the 'MASS' package (Venables & Ripley, 2002). Effects were assessed by comparing nested models using likelihood ratio tests (LRT) and α=0.05.

5.5 Results

Both LCM analyses converged with Gelman-Rubin statistics being ≤ 1.01 for all parameters estimated, and visual assessment confirmed good mixing of chains. All analyses in results, discussion, tables and figures relate to Model–1 unless otherwise specified. For individual tests, DSe (mean, 95% CI) was higher for histology (0.76, 0.68–0.85) than heart smear (0.61, 0.54–0.68), with qPCR intermediate (0.69, 0.61–0.77)

(Figure 21, Table 22). DSp was high for both histology and qPCR (0.93, 0.84–0.99), but relatively low for heart smear (0.60, 0.45–0.73) (Figure 21, Table 22).

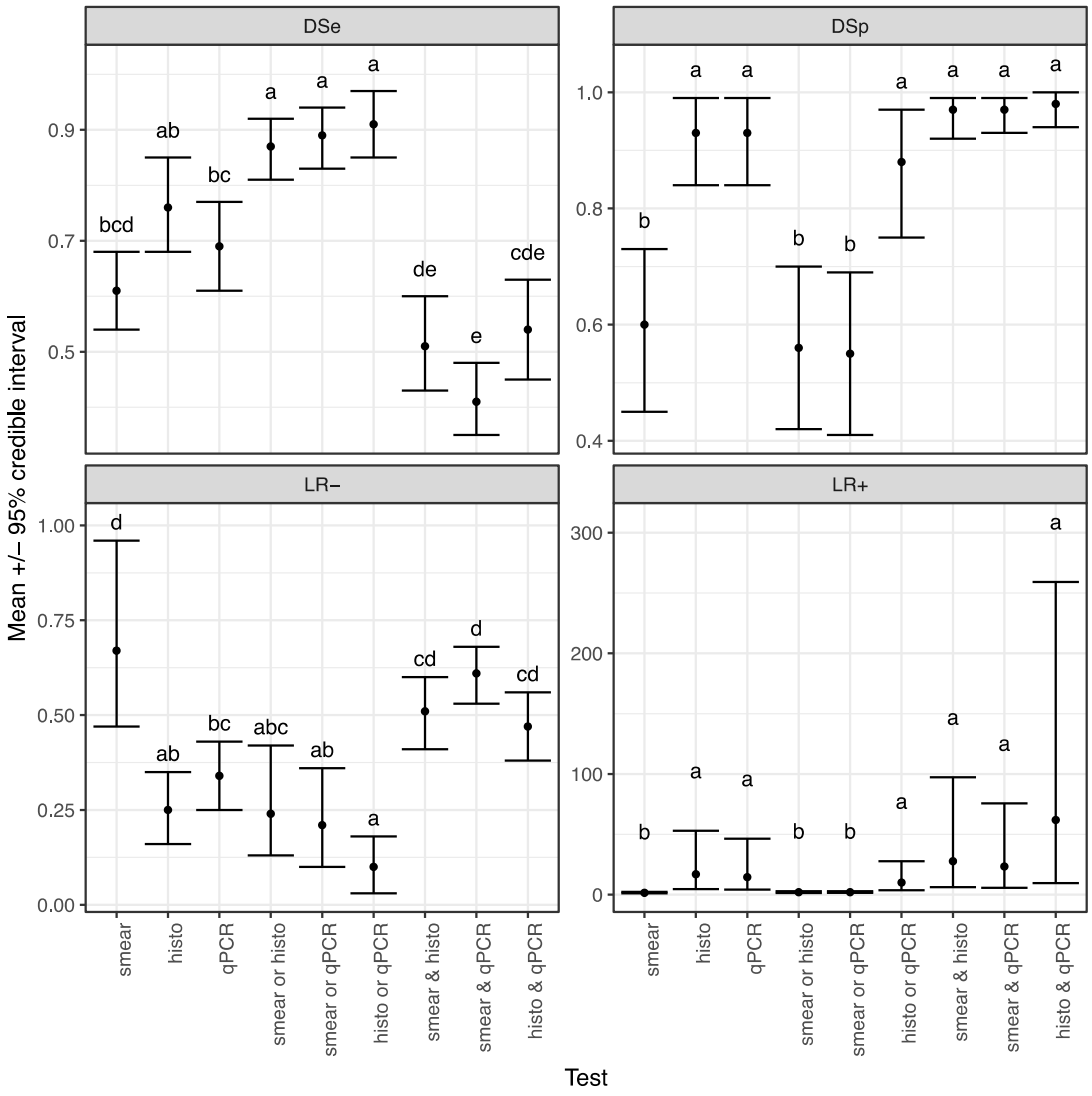

Figure 21. Latent Class Model calculated mean and ± 95% credible interval differences for diagnostic sensitivity (DSe) and specificity (DSp) and the likelihood ratio of positive (LR+) and negative (LR-) test results of single and combined diagnostic tests (where, smear=heart smear; histo=histology; qPCR=quantitative PCR) including both AND-rule (two diagnostic tests, both positive=positive) and OR-rule (two diagnostic tests, either positive=positive) case definitions.

Table 22. The diagnostic sensitivity (DSe) and specificity (DSp) of each diagnostic test for *Bonamia exitiosa* (heart smear, histology and qPCR) from the Latent Class Model with different combinations of parallel tests (OR-rule and AND-rule case definitions).[† ‡ §]

	Case definitions (95% credible intervals)								
	Single diagnostic tests			OR-rule (2 diagnostic tests, either positive = positive)			AND-rule (2 tests, both positive = positive)		
	Smear	Histo	qPCR	Smear/Histo	Smear/qPCR	Histo/qPCR	Smear/Histo	Smear/qPCR	Histo/qPCR
Diagnostic Sensitivity (DSe)	0.61^{bcd} (0.54–0.68)	0.76^{ab} (0.68–0.85)	0.69^{bc} (0.61–0.77)	0.87^{a} (0.81–0.92)	0.89^{a} (0.83–0.94)	0.91^{a} (0.85–0.97)	0.51^{de} (0.43–0.60)	0.41^{e} (0.35–0.48)	0.54^{cde} (0.45–0.63)
	0.63^{bc} (0.56–0.70)	0.77^{ab} (0.68–0.86)	0.62^{bc} (0.54–0.70)	0.88^{a} (0.82–0.93)	0.86^{a} (0.81–0.92)	0.90^{a} (0.82–0.96)	0.52^{cd} (0.44–0.62)	0.38^{d} (0.32–0.46)	0.49^{cd} (0.41–0.59)
Diagnostic Specificity (DSp)	0.60^{b} (0.45–0.73)	0.93^{a} (0.84–0.99)	0.93^{a} (0.84–0.99)	0.56^{b} (0.42–0.70)	0.55^{b} (0.41–0.69)	0.88^{a} (0.75–0.97)	0.97^{a} (0.92–0.99)	0.97^{a} (0.93–0.99)	0.98^{a} (0.94–1.00)
	0.63^{bc} (0.49–0.76)	0.93^{a} (0.83–0.98)	0.93^{a} (0.85–0.99)	0.59^{c} (0.44–0.72)	0.59^{c} (0.45–0.73)	0.88^{ab} (0.75–0.96)	0.97^{a} (0.92–0.99)	0.98^{a} (0.94–1.00)	0.98^{a} (0.95–1.00)
Likelihood ratio of positive test result (LR+)	1.56^{b} (1.04–2.35)	16.92^{a} (4.63–53.00)	14.52^{a} (4.14–46.37)	2.02^{b} (1.43–2.89)	2.04^{b} (1.47–2.88)	10.06^{a} (3.68–27.64)	27.72^{a} (6.19–97.31)	23.35^{a} (5.68–75.64)	61.92^{a} (9.52–259.16)
	1.79^{b} (1.16–2.76)	15.69^{a} (4.44–47.19)	14.00^{a} (3.99–44.54)	2.20^{b} (1.52–3.24)	2.19^{b} (1.51–3.25)	9.53^{a} (3.58–25.22)	30.14^{a} (6.47–98.84)	25.39^{a} (6.06–87.19)	59.27^{a} (9.35–251.27)
Likelihood ratio of negative test result (LR-)	0.67^{d} (0.47–0.96)	0.25^{ab} (0.16–0.35)	0.34^{bc} (0.25–0.43)	0.24^{abc} (0.13–0.42)	0.21^{ab} (0.10–0.36)	0.10^{a} (0.03–0.18)	0.51^{cd} (0.41–0.60)	0.61^{d} (0.53–0.68)	0.47^{cd} (0.38–0.56)
	0.59^{de} (0.42–0.84)	0.25^{ab} (0.15–0.35)	0.41^{bcd} (0.31–0.50)	0.21^{ab} (0.10–0.37)	0.24^{abc} (0.12–0.39)	0.12^{a} (0.04–0.20)	0.49^{cde} (0.39–0.58)	0.63^{e} (0.55–0.70)	0.52^{de} (0.42–0.60)

† Different superscripts denote differences from 0 at a 5% level.

‡ Values in bold were from Model–1, where indeterminate (one positive and one negative within duplicate) qPCR samples were deemed as positive and non-bold values were from Model–2, where indeterminate qPCR samples were deemed as negative.

§ Where: Smear=heart smear, histo=histology, qPCR=quantitative PCR.

DSe of test combinations using the OR-rule were higher than the single smear and qPCR, but not higher than histology alone (Figure 21, Table 22). Given high DSp for histology and qPCR individually, all AND-rule combinations had very high DSp, especially histology/qPCR (0.98, 0.94–1.00). All combinations had higher DSe than heart smears (Figure 21, Table 22).

Covariance of 0.04 (95% CI: 0.02–0.06) or correlation of 0.19 (95% CI: 0.11–0.26) was found between heart smear and histology for the infected case (if one test positive, other likely positive) and there was covariance (0.01, 0.00–0.04) or correlation (0.2, 0.02–0.38) between the histology and qPCR tests for the uninfected case (if one test negative, other likely negative) (Table 23).

Table 23. The covariance between tests and 95% credible intervals from the Latent Class Model (LCM). Upper diagonal shows covariance of tests for infected case and lower diagonal shows covariance of tests for uninfected case.†

		Test 2		
		Smear	Histo	qPCR
Test 1	Smear		0.04 (0.02–0.06)*	-0.01 (-0.03–0.01)
	Histo	0.00 (-0.01–0.02)		0.01 (-0.01–0.03)
	qPCR	0.00 (-0.02–0.01)	0.01 (0.00–0.04)*	

†Where: Smear=heart smear, histo=histology, qPCR=quantitative PCR.

*Different from 0 at a 5% level.

For the *B. exitiosa* tests applied individually, LR+ (mean, 95% CI) was higher for histology (16.92, 4.63–53.00) and qPCR (14.52, 4.14–46.37) than heart smear (1.56, 1.04–2.35) (Figure 21, Table 22). LR- for histology (0.25, 0.16–0.35) and qPCR (0.34, 0.25–0.43) were lower than heart smear (0.67, 0.47–0.96) (Figure 21, Table 22).

All AND-rule test combinations had higher LR+ than the smear/histology (2.02, 1.43–2.89) and smear/qPCR (2.04, 1.47–2.88) OR-rule test combinations and heart smear (1.56, 1.04–2.35) (Figure 21, Table 22). LR- was lower for the histology/qPCR OR-rule test combination (0.10, 0.03–0.18) than all AND-rule test combinations and qPCR (0.34, 0.25–0.43) and heart smear (0.67, 0.47–0.96) (Figure 21, Table 22).

PPV was higher for all AND-rule test combinations, histology and qPCR than the single heart smear test and smear/histology and smear/qPCR OR-rule test combinations (Figure 22). In particular, the histology/qPCR AND-rule combination had high PPV (>0.85), even at low prevalence (0.25) (Figure 22).

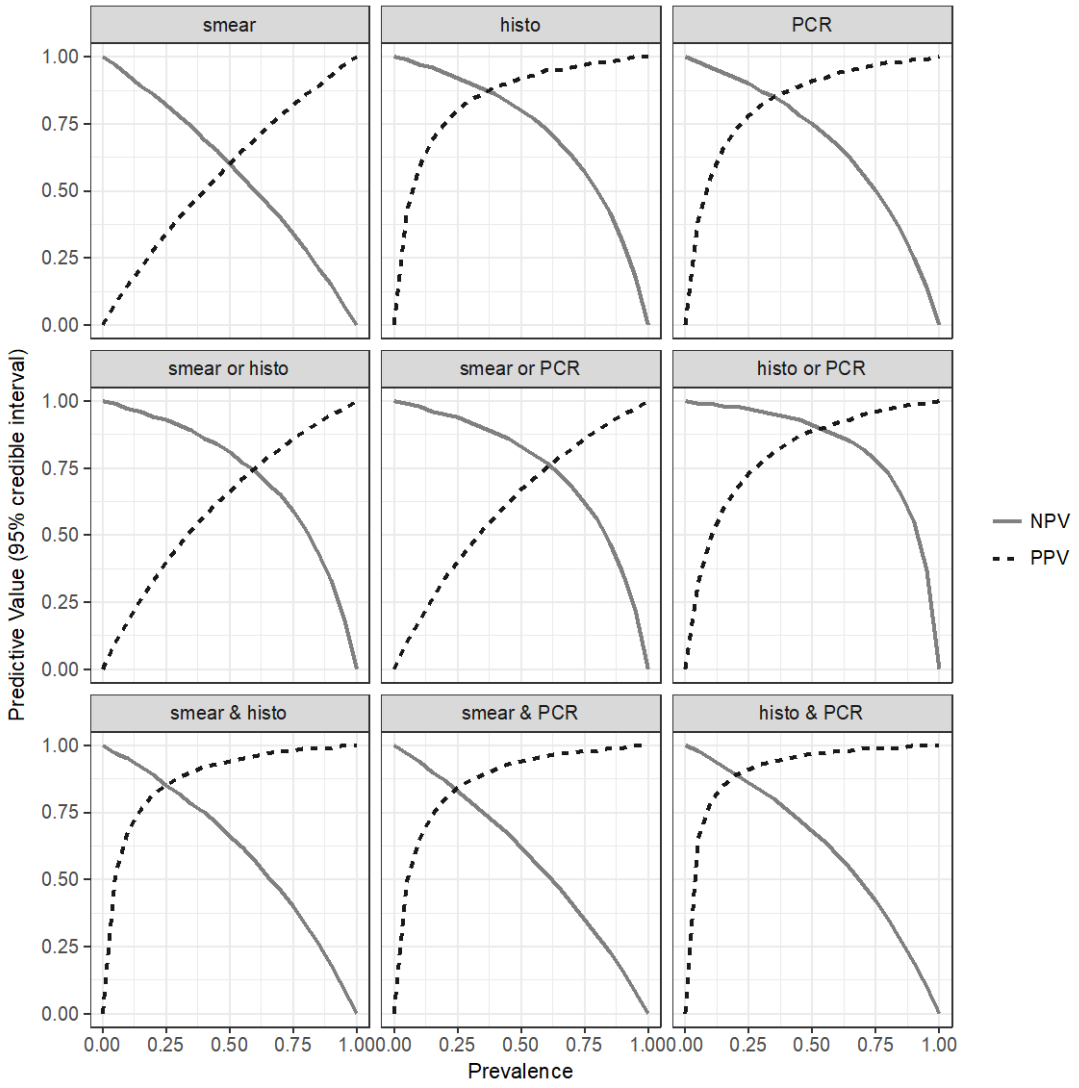

Figure 22. Negative Predictive Value (NPV) and Positive Predictive Value (PPV) with 95% credible intervals of single and combined diagnostic tests for *B. exitiosa* with increasing prevalence.

NPV was higher for all OR-rule test combinations than all single tests and all AND-rule test combinations (Figure 22). In addition to highest NPV for the histo/qPCR OR-rule combination, the PPV also remained >70 at low prevalence (0.25) with similar PPV trends to the single histology and qPCR (Figure 22).

When Model–2 LCM was run, there was decreased predicted sensitivity for the qPCR single test than Model–1, but little change to other estimated test performance parameters (Table 23).

Estimated *B. exitiosa* prevalence for the hatchery, where 150 samples tested negative by both histology and PCR, was 0.017 (0.000–0.053) (mean, 95% CI). For the farm sites, prevalence, as estimated by the LCM, was >50% in *O. angasi* (Table 20) at all sites. Coffin Bay sites had higher prevalence (mean, 95% CI) (site a and b: 0.90, 0.78–0.99), than at Streaky Bay (0.59, 0.46–0.72) (Table 20). *Bonamia exitiosa* was not detected at the hatchery and those data were excluded from further diagnostic test analyses.

Model–1 estimated higher predicted prevalence in Coffin Bay B than Model–2 (Table 20).

Intensity from histology was highest at Coffin Bay A (mean, bootstrap CI: 4.07, 2.88–6.21), followed by Coffin Bay B (1.88, 1.69–2.10) and Streaky Bay (1.55, 1.35–1.67) (Table 20). *Bonamia exitiosa* intensities from heart smears were numerically higher than intensities from histology for each site and did not differ between all sites (Table 20).

The GLM showed a significant three-way interaction of site, weight and meat:shell ratio (LRT: χ^2 (2) =11.4, p=0.003) on intensity recorded by histology. No significant effects were found for heart smear intensities (LRT p>0.4).

5.6 Discussion

Bonamia exitiosa occurs broadly in South Australia and we describe DSe, DSp and the prevalence of *B. exitiosa* in farmed *O. angasi*. South Australian *O. angasi* are infected with *B. exitiosa* at high prevalence, but low intensity, consistent with Corbeil et al. (2009) who noted that Australian *Bonamia* infections predominantly display focal lesions with few parasites.

Tests with reliable negative results and high DSe are best for proving freedom (Fegan, 2000). Histology has higher DSe, NPV and lower LR- than heart smear. This is consistent with OIE (2017) recommendations. Diggles et al. (2003) found that heart smear had higher sensitivity than histology for *B. exitiosa* in New Zealand and Lynch, Mulcahy & Culloty (2008) assessed heart smears as more sensitive than PCR or histology. PCR is usually regarded, however, as more sensitive than histology and/or heart smear (Carnegie, Barber, Culloty, Figueras, & Distel, 2000; Diggles et al., 2003; Marty et al., 2006; Michael, Forman, Hulston, Fu & Maas, 2015; Ramilo et al., 2013; Robert et al., 2009). Unrepresentative negative results occur when subsampled DNA for qPCR does not contain the target (Lynch et al., 2008) or because the small physical size of the tissue sample does not contain the parasite. Localised *Bonamia* cell distribution (Diggles et al., 2003) makes sampling an infected section of tissue less likely. We followed Lane et al. (2018a) and Lane (2018b) and used mantle, gill and the heart tissue remaining from the smear as the tissues for qPCR. Using a broader range of tissues and analysing each sample in quadruplicate (8 μL DNA per sample tested) mitigates the likelihood of missing localised infections. Mantle and heart tissue are sites of *Bonamia* infection but these tissues do not cause PCR inhibition, unlike digestive gland (Schrader et al., 2012). Proper controls and process quality assurance mitigates negative effects of poor sample preparation (Diggles et al., 2003).

Heart smears work better when oysters have high intensity infections (Flannery et al., 2014; Lynch et al., 2008) but not for low intensity (Flannery et al., 2014). Our oysters generally had low *B. exitiosa* intensity. Infected oysters had *B. exitiosa* concentrated in the digestive gland epithelia but few cells in the hemolymph, gills and mantle (Hine, 1991; Hine & Wesney, 1994), indicating that the infection was not systemic (Corbeil et al., 2009). If there is an intensity threshold below which infection is not systemic (Corbeil et al., 2009) low heart smear DSe is likely to be provided by analysis of such samples.

Combining any pair of tests with the OR-rule improves DSe and is better for demonstrating freedom than smears or qPCR alone. Flannery et al. (2014) and Lynch et al. (2008) also found higher sensitivity for combined tests than single tests for *Bonamia*. High DSe contributes to OR-rule combinations having lower LR- and higher NPV while AND-rule test combinations have lower DSe and higher LR- and are less suitable than OR-rule test combinations for

demonstrating freedom (Weinstein et al., 2005). Histology did not differ in DSe or LR- to all OR-rule test combinations and both are suitable for demonstrating freedom. It is important to consider costs when designing surveillance plans. Single tests can reduce survey cost, but more expensive OR-rule combinations of tests improve DSe and LR-. To limit cost, a second diagnostic test could be performed only for samples that are initially assessed as negative by histology.

Histology and qPCR had higher DSp, PPV and LR+ than the single heart smear test, supporting these as better tests for assessing prevalence or maximising detection where reliable positive results are necessary and high DSp is favoured (Fegan, 2000). High specificity of both histology and qPCR is consistent with findings for *B. ostreae* and/or *B. exitiosa* in *O. edulis* with low (0.5–11.1%) (Marty et al., 2006) or high (57–89%) prevalence (Ramilo et al., 2013). Despite low DSp, PPV and LR+, heart smears have advantages for screening large oyster numbers because they can be made and assessed rapidly and are inexpensive (Diggles et al., 2003).

While AND-rule test combinations increase DSp, assessment of prevalence was not improved by combining tests compared to histology or qPCR which have high DSp alone. OR-rule test combinations generally have worse test performance than AND-rule combinations (Weinstein et al., 2005), except the histo/qPCR OR-rule combination which gave similar DSp, PPV and LR+ to AND-rule combinations and individual tests. Higher DSp for this OR-rule test is due to high DSp in both histology and qPCR alone. For assessing *B. exitiosa* prevalence, in this study single histology or qPCR tests were adequate, yet combined tests were more informative as each test brought unique data with different benefits.

Histology is the gold standard test for *Bonamia* (OIE, 2017) and we found that it had the highest DSe and high DSp for a single test. All tests are imperfect but histology can measure intensity, provides information on other pathogens, and provides indicators of general health and reproductive condition and a permanent record (Diggles, 2003). Training diagnosticians to identify *Bonamia* in heart smears and histology is, however, more difficult than training for qPCR (Balserio et al., 2006).

Compared to histology, PCR has the benefit of speed (Marty et al., 2006), but can be more costly. PCR can exclude other pathogens that are morphologically similar (Stokes & Burreson, 2001), but PCR cannot assess pathogen viability or distinguish between infection and disease. When choosing a test, in addition to the purpose of testing, it is important to consider what information additional to presence/absence is needed. Combining molecular and microscopy techniques provides the best combination of information about the disease status of a case (Aranguren & Figueras, 2016; Burreson & Ford, 2004).

Despite low intensity infection, histology used mantle and gill as a component of its analyte, which is also used in qPCR and could explain covariance for the un-infected case between qPCR and histology. Heart smear and histology used different tissue analytes but both visualise haemocytes (the site of *Bonamia* infection) which could explain covariance for the infected case between these diagnostic tests. Covariance between two tests can occur when the two tests are heavily dependent on infection intensity (Dendukuri & Joseph, 2001) and at higher intensities these results may be more pronounced.

Our study confirmed that *B. exitiosa* has >50% prevalence on *O. angasi* farms in SA. Using all three tests facilitated understanding *B. exitiosa* infection dynamics and highlights the need

for developing an understanding of *B. exitiosa* seasonality in *O. angasi*. *Bonamia* spp. infections can have marked seasonality but these differ between geographic regions and hosts and exhibit contrasting patterns of prevalence and intensity (e.g. see Hine, 1991; Carnegie et al., 2008). While OIE (2017) recommends sampling in January-April based on peak prevalence in *O. chilensis* in New Zealand, peak intensity can occur in August (Hine, 1991) and peak prevalence varies widely (OIE, 2017). There are no data on *B. exitiosa* seasonality in Australia, and in the absence of such data, understanding test performance is critical, particularly given that *Bonamia* infections often occur with low prevalence and intensity and are therefore difficult to detect (Corbeil et al., 2009). Prevalence of *B. exitiosa* was higher in Coffin Bay than Streaky Bay, and the highest parasite intensity was found at Coffin Bay. A *Bonamia exitiosa* intensity was low at all sites (intensity averages were classed grade–2–light infection) in comparison to European farm intensities which were equivalent to our grade–5–systemic infection (Culloty, Cronin & Mulcahy, 2004). *Bonamia* seasonality in New Zealand is further complicated by outbreaks having occurred over a long period with epizootics occurring every 20–30 years (Hine, 1996).

The significant interaction term in the GLM for histology intensity was due to the highest histology intensities of >10 cells consistently occurring in above average-size oysters from Coffin Bay A with low meat:shell ratios. Higher intensity infection and lower condition in older oysters (>2 years) was reported by Arzul et al. (2011), Culloty and Mulcahy (1996) and Robert, Borel, Pichot and Trut (1991) although Arzul & Carnegie (2015) outlined that disease can affect oysters less than one year old. Growth and age at harvest of *O. angasi* differs substantially from *O. edulis* and *O. chilensis*; the larger animals we sampled were 20–-22 months old and were sampled at harvest. This is the typical maximum age for harvest in South Australia, hence in many oyster farming areas, sampling stock over 24 months of age is impossible. These data support the notion, nevertheless, that for *Ostrea* spp., including *O. angasi*, older and larger oysters generally have higher intensity *Bonamia* infections than smaller oysters. It is also extremely important to understand test performance in young animals for screening stock from hatcheries for translocation to areas where *Bonamia* has not been detected.

Differences in *B. exitiosa* prevalence and intensity between farms in SA may be associated with lower oyster density and proximity (Arzul & Carnegie, 2015; Lallias et al., 2008) of *O. angasi* and *C. gigas* in Streaky Bay (16 leases) than in Coffin Bay (141 leases) (PIRSA, 2017). Given that *C. gigas* is probably a host of *Bonamia* (see Hill et al., 2014; Lynch et al., 2010), large populations of Pacific oysters may be important reservoirs of *Bonamia* in the environment. No *B. exitiosa* was detected in *O. angasi* spat (via histology or qPCR) from the hatchery site, despite broodstock having been sourced from infected areas. The true prevalence range estimated by the LCM gives confidence that the hatchery spat are not infected with *B. exitiosa*.

We ran two different LCMs, defining indeterminate cases for qPCR as either positive (Model–1) or negative (Model–2). Higher DSe estimated by Model–1 for qPCR is expected as the number of positives was increased. In addition, higher prevalence estimated by Model–1 in Coffin Bay B is due to a large number of indeterminate cases at that site. This highlights the importance of case definition of indeterminate qPCR cases. In a population that is assumed to be free of infection, an indeterminate case should elicit further testing, as there is greater consequence for future management decisions in defining an indeterminate case as positive.

The first step for confirming indeterminate cases is to re-test another subsample of the same DNA. Testing different oyster tissues from the same individual in parallel could also further decrease false negatives (Carnegie et al., 2000).

These data support understanding surveillance approaches for different oyster populations. Australian *O. angasi appear to be characterised by low B. exitiosa* intensity. Given that low intensity infections are likely to be found during the early phases of pathogen establishment and in juvenile oysters, these approaches are important for early detection and health certification prior to translocation, in addition to surveillance programs. Characterising diagnostic methods facilitates understanding changes in host-parasite dynamics, and characterising *Bonamia* infections in different farming regions can aid regulators and oyster growers to make decisions for development and expansion of oyster farming industries.

5.7 Conclusion

South Australian *B. exitiosa* infections mostly have low parasite abundance. Histology is the best single test for these infections, while qPCR is also suitable as a single test for assessing prevalence. Heart smears are lower cost and quicker, which can be advantageous in practice. Histology combined with PCR in the OR definition maximises diagnostic performance. The DSe and DSp data developed here can be used to plan surveillance for *B. exitiosa*.

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6. SARDI - Infection of *Ostrea angasi* with *Bonamia exitiosa* by cohabitation

6.1 Summary

Infection dynamics of *Bonamia* are poorly understood, particularly of *B. exitiosa* in *Ostrea angasi*. We used a cohabitation infection model and showed that as infection occurs, abundance builds, and mortality due to clinical *B. exitiosa* infection occur rapidly. Host death is not required for transmission. This infection system can be used to develop a system by which oysters can be assessed for susceptibility to *Bonamia* and could form the basis for an assessment process to develop resistant families of *O. angasi* to further develop the industry in Australia.

6.2 Introduction

Infection dynamics of *Bonamia* exitiosa in Australia are poorly understood. *Bonamia* spp. transmit directly (Arzul & Carnegie, 2015; Culloty et al., 1999; Engelsma et al., 2014). A spore was described for *B. perspora* by Carnegie et al. (2006) but the life history of the parasite outside and between its hosts is not documented for other *Bonamia* spp. Development of an infection model can facilitate obtaining information on the effect of temperature, salinity, and other conditions that contribute to infection and pathogenicity and understanding the lifecycle (Carnegie et al., 2008). *Bonamia* can be detected within weeks of deployment in summer and early fall in the USA (Carnegie et al., 2008) but infection appears to occur more slowly than reported for *B. ostreae* in Europe (Elston, Farley, & Kent, 1986; Lallias et al., 2008). We suspected, however, that infection may occur rapidly and that field infection dynamics are influenced by environmental factors rather than inherent parasite characteristics.

6.3 Objectives

To facilitate understanding of the infection dynamics of Southern Australian *B. exitiosa*, we evaluated the rate of infection acquisition of the parasite in *Ostrea angasi*. We began this study to investigate *B. exitiosa prevalence and intensity over time. This study investigated exposure time needed to infect O. angasi*, changes in prevalence of *B. exitiosa* infection and mortality with ongoing exposure.

6.4 Methods

The experimental system comprised of eight 52 L volume (65 cm L x 41 cm W x 27.5 cm H) plastic tanks, with continuous aeration. Four tanks were used in the exposed treatment; 10 adult donor *O. angasi* (72.03 ± 2.84 g) from a Coffin Bay farm with high prevalence (Bayesian calculated prevalence, credible intervals: 0.90, 0.78–0.99) (Buss, Wiltshire, Prowse, Harris, & Deveney, 2018) were cohabited with 300 recipient juvenile *O. angasi* (mean weight ± SE: 1.61 ± 0.03 g) sourced from the South Australian Research and Development Institute (SARDI) SA Aquatic Sciences Centre (SAASC) Mollusc Hatchery (West Beach, Adelaide, South Australia). In addition, four tanks with 300 juvenile *O. angasi* per tank were used for the control treatment. Testing by real-time PCR and histology found no *B. exitiosa* in the recipient animals (Bayesian calculated prevalence, credible intervals: 0.017, 0.000–0.05, Buss et al., 2018).

Exposure began on 26 March 2017. Oysters were monitored every second or third day, provided a 100% water exchange and fed 1.5 L of a 2 x 10^6 cells/mL mixed culture of *Chaetoceros muelleri, Skeletonema costatum and Pavlova lutheri* per tank. Tank temperature, DO and nitrogen compounds were monitored. Each tank was also assessed for mortalities. All mortalities containing tissue were sampled. Some mortalities were too autolysed to sample. Recipient oysters containing tissue were sampled for *Bonamia* testing at day 10, 21 and 40 (38 juveniles per tank). From the original samples, 44 recipients were sampled for heart smear and a subset of 10 were sampled for histology at day 10, day 21 and 40. All mortalities (n=174) were analysed by heart smear and all suitable mortalities (n=10) were analysed by histology. All adult donor oysters were sampled at the end of the experiment and analysed for heart smear and 10 sub-sampled for histology. All intensities were analysed using the scoring system outlined by Buss et al. (2018). Sampled recipients were replaced. The experiment ended on 5 May 2017.

Parasitology terminology used is consistent with Bush et al. (1997). Apparent prevalence was defined as the proportion of individuals that were positive for a single test or a combination of tests, with the assumption that each test is perfect. Estimated prevalence did not assume that each test was perfect and instead used priors to inform a Bayesian model and calculate more realistic estimates of prevalence. Prevalence (apparent and estimated) was based on histology and heart smear test results in the parallel 'AND-rule' case definition to maximise diagnostic specificity (DSp) (see Buss et al., 2018), unless otherwise stated; extra GLM analyses were done for heart smear prevalence alone (see Figures 24–27).

Estimated prevalence with credible intervals were estimated using a Bayesian JAGS code modified from the R Package "prevalence" (Devleesschauwer et al., 2015). JAGS was run using the "R2jags" package (Su & Yajima, 2015) in R (R Core Team, 2017). For this analysis, posterior predictions from DSe, DSp and conditional covariance for a positive or negative disease status calculated in Buss et al. (2018) were used to inform priors for the two tests used in this study, histology and heart smear. Within the new model, the following beta priors: (113, 72) and (27, 18) were used for heart smear DSe and DSp respectively. In addition, the beta priors: (75, 23) and (40, 3) were used for histology DSe and DSp respectively. Overlapping credible intervals signified no difference.

Bonamia exitiosa infection intensity from histology and heart smears for each treatment were analysed using Quantitative Parasitology 3.0 (Reiczigel & Rózsa, 2005) using 95% bootstrap confidence intervals for mean intensities with 2000 replicates. Survival was assessed using Kaplan-Meier analyses with Log-Rank and Breslow tests (IBM SPSS version 23 for Macintosh). Overlapping confidence intervals signified no difference.

Generalised Linear Models (GLM) with binomial distribution were used to assess prevalence for exposed animals. Prevalence was assessed for live juveniles collected on days 10 and 40. GLMs also assessed the effect of status (live or dead) and age (juvenile or adult) on heart smear prevalence on days 22–40 and the effect of status on heart smear prevalence for exposed recipient oysters over time (on days 0–10 and on days 22–40). *Bonamia exitiosa* intensity was assessed for exposed animals. GLMs were used to assess histology intensity over time. GLMs were also used to assess heart smear intensities for exposed recipient oysters (dead and alive) over time (on days 0–10 and on days 22–40) and to assess heart

smear intensities for both recipient and donor oysters (dead or alive) sampled on days 22–40. A negative binomial distribution was assumed for intensity analyses, due to over dispersion of data relative to a Poisson distribution. GLMs were run in R using the "stats" package (R Core Team, 2017) and negative binomial GLMs were run in the "MASS" package (Venables & Ripley, 2002).

6.5 Results

Water quality was normal throughout the trial: DO%: 97.88% (SE ± 0.36), DO mg/L 7.74 (SE ± 0.10), ammonia: 0 mg/L, salinity: 38 ppt, temperature: 16.83 (SE ± 0.58).

The first mortality in recipient oysters was observed on day 12. By day 15 all recipient tanks displayed mortality. On day 21, all samples were gaping mortalities with tissue for recipient tanks and live samples for control tanks. On day 10 and 40 all samples were live samples. Exposed recipient oysters had significantly lower survival than control ($p<0.05$, Figure 23). The largest decrease in survival in recipient oysters occurred on day 21 (by approximately 50%) after which mortality continued but at a reduced rate (Figure 23). By day 40, survival for recipient oysters varied from 8.8–14.9%. The trial was planned to run to day 120 but was terminated due to an inadequate number of remaining animals. There was one mortality in a control tank at day 21 which was negative for *B. exitiosa* by histology and heart smear. All other control tanks had 100% survival across the experiment.

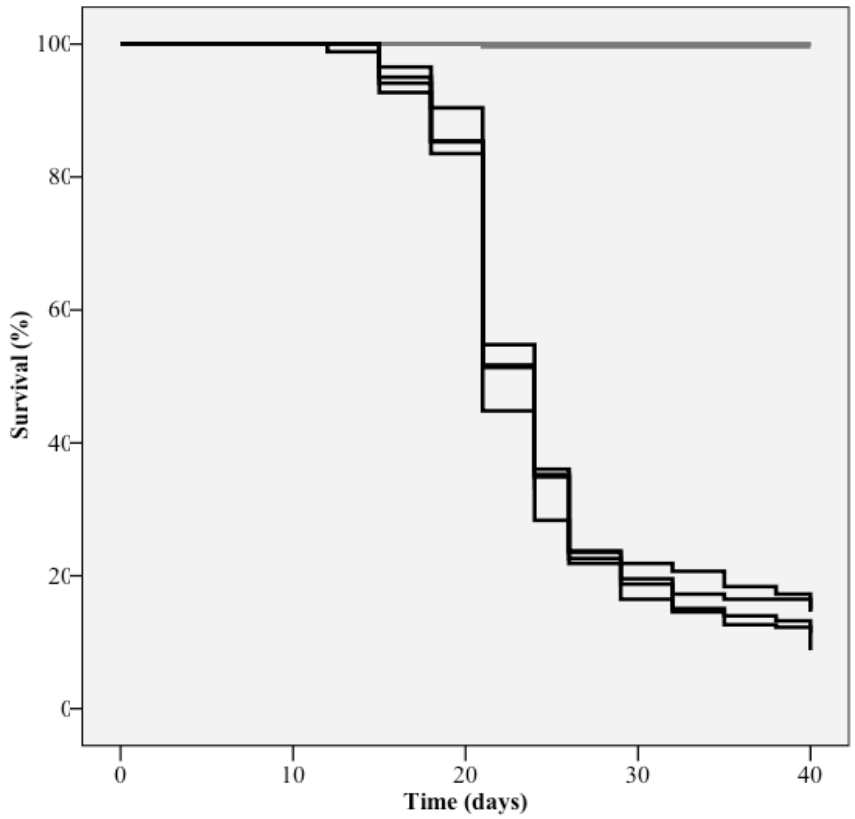

Figure 23. Kaplan-Meier survival curve for recipient *Ostrea angasi* juveniles over 40 days in exposed tanks (black lines) and control tanks (grey lines). Survival for all control tanks was significantly less than all exposed tanks ($p<0.05$)

All donor oysters sampled during the trial, either as mortalities during the experiment or sampled at its conclusion were infected with *B. exitiosa* (Table 24). The mortality from the control treatment was negative for *B. exitiosa*.

Live recipient oysters had significantly higher apparent prevalence on day 40 than on day 10 **(**CHI: $\chi 2$ (1) =12.28, p=4.59e-4). Recipient oysters sampled on days 22–40 had significantly lower apparent heart smear prevalence than donor oysters sampled on days 22–40 (CHI: $\chi 2$ (1) =35.56, p=2.48e-9) (Figure 24). Dead oysters sampled on days 22–40 had significantly lower apparent heart smear prevalence than live oysters sampled on days 22–40 (CHI: $\chi 2$ (1) =36.27, p=1.71e-9) (Figure 25). Recipient oysters sampled on days 22–40 had significantly higher apparent heart smear prevalence than recipient oysters sampled on days 0–10 (CHI: $\chi 2$ (2) =13.24, p=1.34e-3) (Figure 26). Live recipient oysters had significantly higher apparent heart smear prevalence than recipient mortalities sampled on days 0–10 and days 22–40 (CHI: $\chi 2$ (1) =36.23, p=1.75e-9) (Figure 27). Control oysters had 0% apparent prevalence across the experiment (Table 24). Recipient oysters had 40%, 94.11% and 100% apparent prevalence on day 10, 21 and 40 respectively (Table 24). Recipient oysters sampled on days 0–10 (mean, 95% credible interval: 0.51, 0.18–0.64), had lower estimated prevalence than recipient oysters sampled at any other time or donor oysters (Table 24).

Live recipient oysters had significantly higher histology intensities on day 40 than day 10 **(**CHI: $\chi 2$ (1) =22.38, p=2.24e-6) (Figure 26). Living and dead donor oysters sampled on days 22–40 had significantly higher heart smear intensities than recipient oysters sampled on days 22–40 (CHI: $\chi 2$ (1) =166.65, p=2.00e-16) (Figure 27). Live and dead recipient oysters sampled on days 22–40 had significantly higher heart smear intensities than live and dead recipient oysters sampled on days 0–10 (CHI: $\chi 2$ (2) =7.15, p=2.80e-2) (Figure 28).

Recipient oysters sampled on days 0–10 had lower histology intensities (mean, bootstrap CI: 5.50, 2.75–8.00) than recipient oysters sampled at any other time or donor oysters (Table 24).

On day 40, two control oysters were positive for *B. exitiosa* by heart smear with low heart smear intensity (very light to light infection grade) (Table 24). These same samples were negative by histology.

Both heart smear and histology *B. exitiosa* intensities increased over time for exposed treatments (Table 24). Donor oysters had higher histology and heart smear intensities than exposed recipient juveniles sampled on days 0–10. (Table 24). All control oysters had zero heart smear intensity except on days 22–40 (1.50, 1.00–1.55) (Table 24).

Table 24. Size data, apparent prevalence, Bayesian estimated prevalence with credible intervals (calculations used known DSe, DSp and covariance values for heart smear and histology as priors) and mean intensity with confidence intervals (calculated through Quantitative Parasitology) of *Bonamia sp*. in *Ostrea angasi* from heart smears and histology sampled on days 0–10, days 11–21 and days 22–40 in control and exposed treatments. Confidence intervals could not be calculated when values were constant. Intensity cell counts were capped to 30 cells maximum

		Size data					Bayesian	Quantitative Parasitology (bootstrap, 95%)	
Sample period/s	**Treatment**	**Weight (g) (mean±SE)**	**Shell length (mm) (mean±SE)**	**Meat:shell ratio (%) (mean±SE)**	**n**	**Apparent prevalence (%)**	**Estimated prevalence (credible intervals)**	**Mean heart smear intensity cell count (confidence intervals)**	**Mean histology intensity cell count (confidence intervals)**
Days 0–10	**Control**	1.54±0.20	22.98±1.51	1.25±0.16	10	0	0.11 (0.00–0.15) [c]	0	0
Days 11–21	**Control**	1.74±0.30	23.69±1.63	1.37±0.23	10	0	0.11 (0.00–0.15) [c]	0	0
Days 22–40	**Control**	1.89±0.17	25.57±0.68	1.47±0.14	10	0	0.11 (0.00–0.15) [c]	1.50 (1.00–1.55) [c]	0
Days 0–10	**Exposed**	1.32±0.28	20.61±1.50	1.07±0.23	10	40	0.51 (0.18–0.64) [b]	5.30 (4.00–6.90) [b]	5.50 (2.75–8.00) [b]
Days 11–21	**Exposed**	1.12±0.10	21.71±0.82	0.99±0.09	17	94.11	0.93 (0.78–0.98) [a]	15.40 (11.40–20.30) [a]	19.20 (15.00–23.40) [a]
Days 22–40	**Exposed**	1.70±0.24	22.75±1.44	1.39±0.20	16	100	0.94 (0.79–0.98) [a]	16.20 (12.70–20.70) [a]	19.70 (15.00–24.30) [a]
Days 0–40	**Donor**	72.20±3.89	74.65±1.12	60.50±3.60	18	100	0.95 (0.82–0.98) [a]	24.20 (18.40–27.70) [a]	14.10 (10.20–18.30) [a]

† Different superscripts denote differences at a 5% level, with [a] representing the highest value.

‡ Control and exposed treatments included juvenile mortalities and live samples per time period. The adult treatment included live adults sampled on day 40 and any adult mortalities that occurred throughout the trial.

§ Both apparent and estimated prevalence values were calculated using the AND-rule case definition (sample positive, if both heart smear and histology were positive).

¶ Priors for the heart smear and histology tests were derived from Buss et al., (2018). SE: standard error; n: sample number per site.

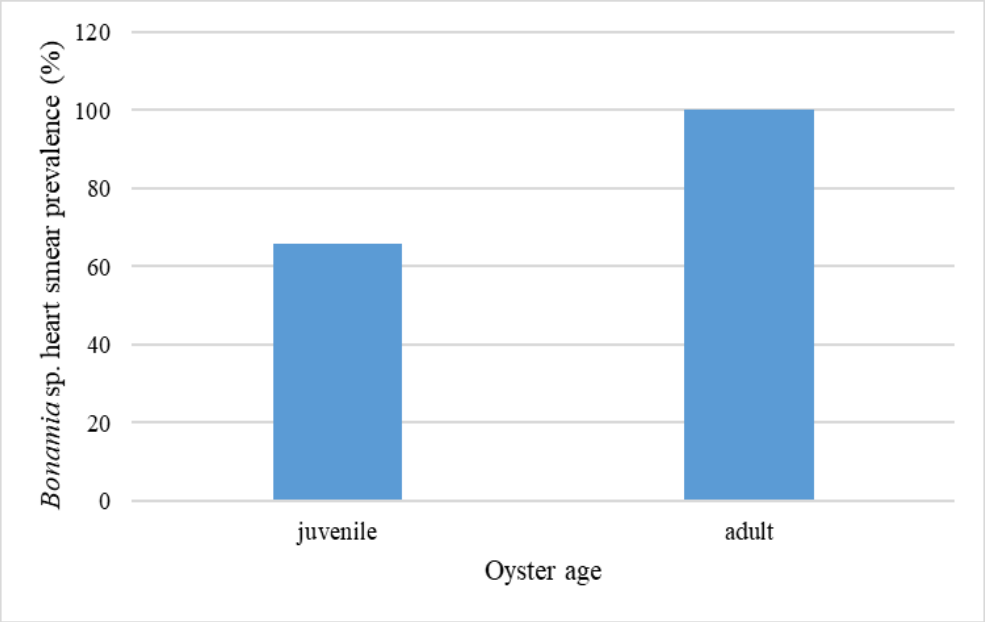

Figure 24. *Bonamia sp.* apparent prevalence from heart smears in recipient juvenile and adult *Ostrea angasi* (mortalities and live samples included) sampled on days 22– 40

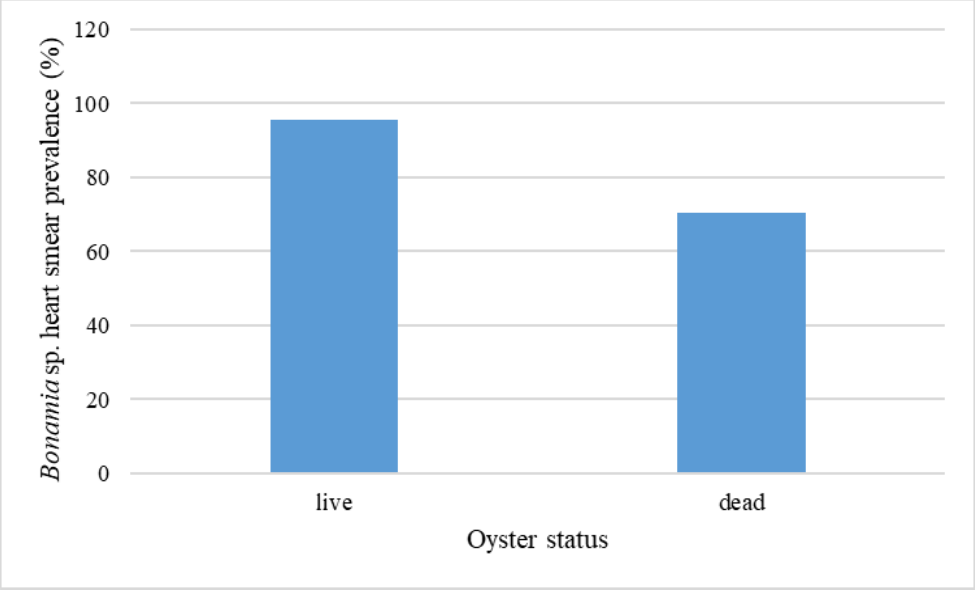

Figure 25. *Bonamia sp.* apparent prevalence from heart smears for dead and live recipient and donor *Ostrea angasi* sampled on days 22–40

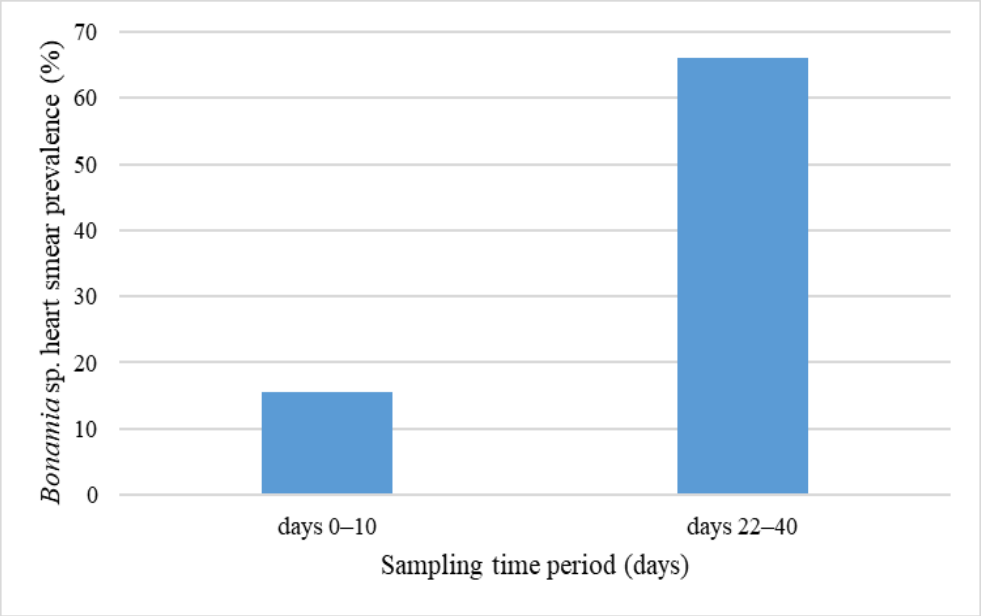

Figure 26. *Bonamia sp.* apparent prevalence from heart smears for dead and live recipient *Ostrea angasi* sampled on days 0–10 and days 22–40

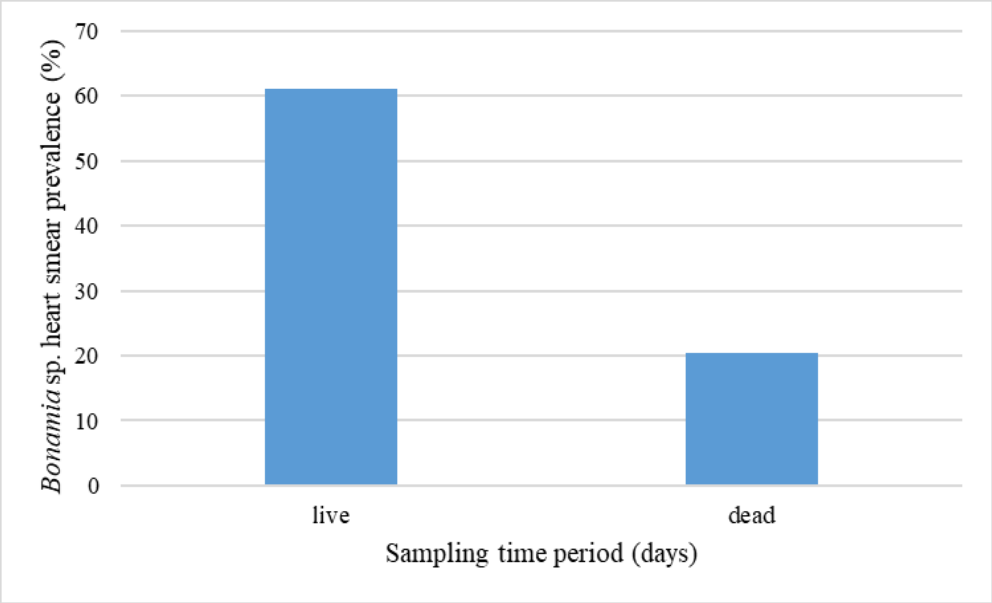

Figure 27. *Bonamia sp.* apparent prevalence from heart smears in dead and live recipient *Ostrea angasi* sampled on days 0–10 and days 22–40

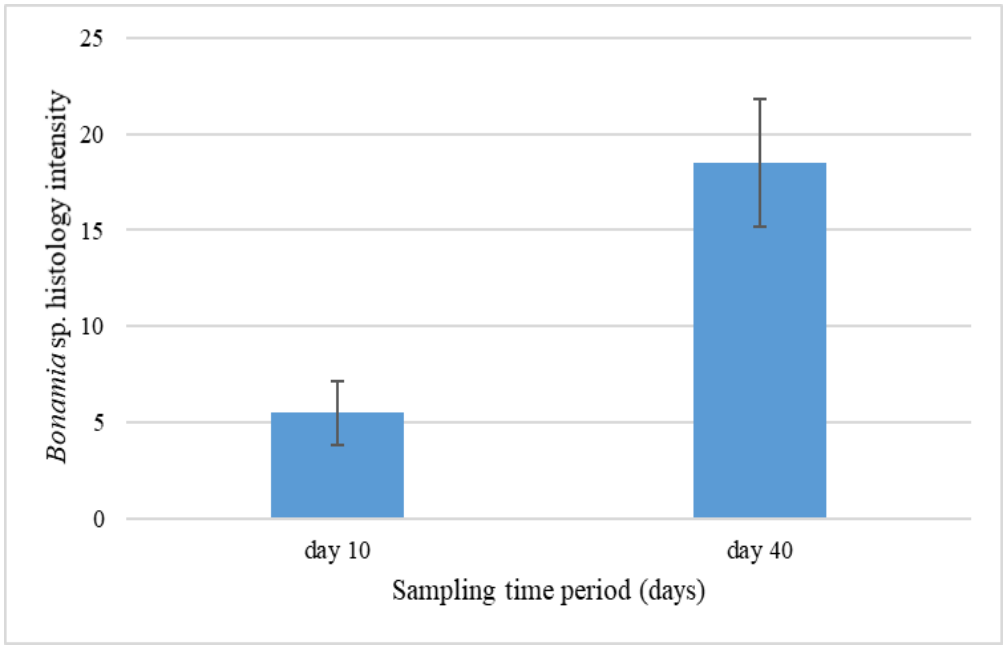

Figure 28. *Bonamia sp.* mean intensity from histology for live recipient *Ostrea angasi* sampled on day 10 and day 40. Mean ± SE

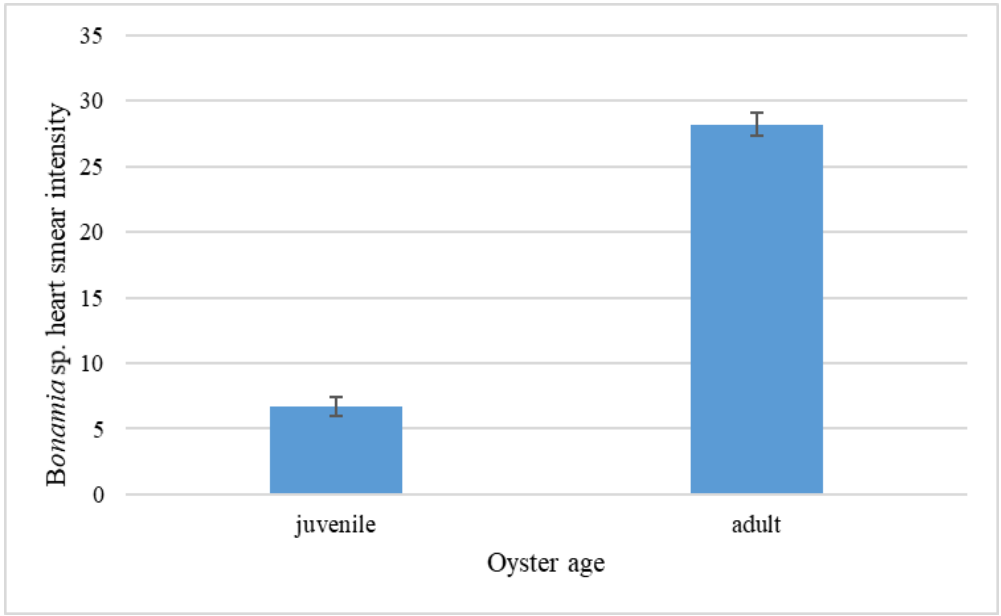

Figure 29. *Bonamia sp.* mean intensity from heart smear for dead and live recipient and donor *Ostrea angasi* sampled on days 22–40. Mean ± SE

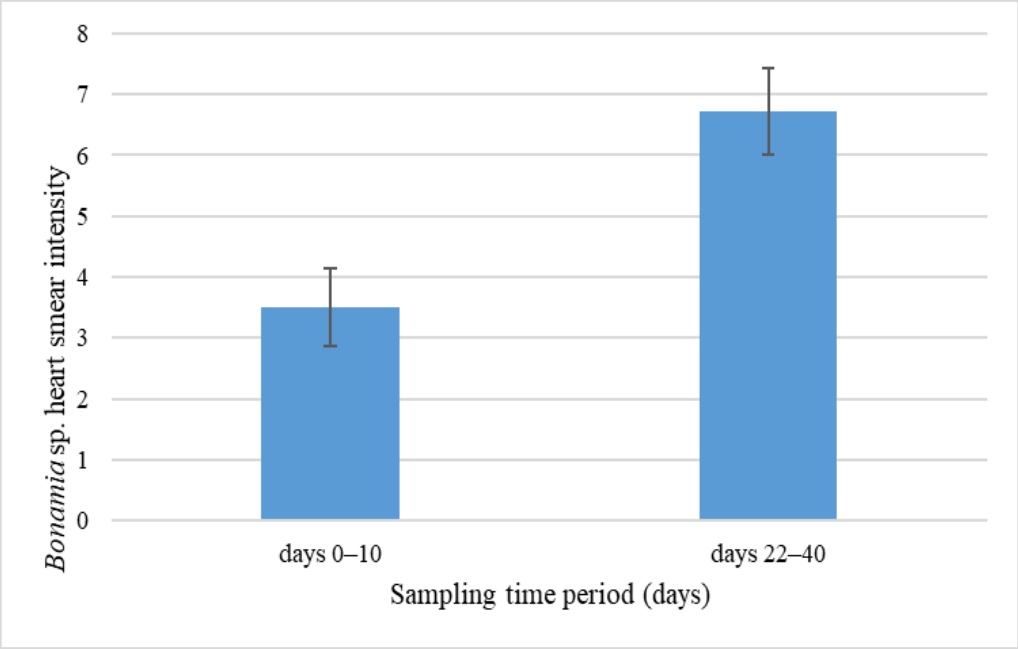

Figure 30. *Bonamia* sp. mean intensity from heart smear for dead and live recipient *Ostrea angasi* sampled on days 0–10 or days 22–40. Mean ± SE

6.6 Discussion

Juvenile *O. angasi* are infected rapidly by *B. exitiosa* Infection occurs more rapidly after exposure than is described for *B. ostreae* in European *O. edulis* where first detection occurs after 2+ months of field exposure in an enzootic area (Lynch, Armitage, Wylde, Mulcahy, & Culloty; Montes, 1991), or after 4 months cohabitation in a laboratory (Lallias et al., 2008). Our recipient oysters became infected within 10 days (Table 24). This is more rapid than occurred in *B. exitiosa* in *C. ariakensis*, where first detection occurred 14 days post exposure with 3.3% prevalence, increasing to 30% prevalence by day 21. When uninfected recipient *C. ariakensis* were cohabited in a laboratory with donor *C. ariakensis* that had been exposed in the field for 35 days prior to cohabitation, the first *B. exitiosa* detection in the recipient oysters occurred after 28 days with 13% prevalence (Audemard et al., 2014). Our donor oysters had been grown in an enzootic area for 20 months and subsequently cohabited in the laboratory for 5 months. It is possible that the system we used with heavily infected adult donor oysters provides greater infection pressure than cohabitation of juveniles of the same age, leading to more rapid parasite infection and proliferation to detectable abundance.

Bonamia exitiosa causes mortality in *O. angasi* with the first deaths with clinical *Bonamia* infection observed 12 days post exposure. This indicates that under conditions favourable for the parasite, disease can manifest rapidly, and mortality in oysters may not be linked to long--term exposure or a slow accumulation of parasites. Rapid and lethal infection suggests *O. angasi* have not co-adapted with *B. exitiosa* over a long evolutionary history, and that the parasite may therefore be a relatively recent introduction to Southern Australia. This is reinforced by surveys in the 1990s failing to find the parasite (Corbeil et al., 2009) in areas

which by 2015/2016 consistently had high *B. exitiosa* prevalence (Buss et al., 2018). Larger *O. angasi* appear to be able to sustain higher *B. exitiosa* intensities than juvenile oysters, compounding the risk in growing areas where age classes are mixed.

Despite the apparent low intensity of field infections, *B. exitiosa* prevalence and intensity increase with duration of exposure. It is likely that sites where Native Oysters are grown with substantial population sizes and high density, but where mortality due to *B. exitiosa* is not observed, are characterised by low exposure due to biotic or oceanographic characteristics. This further suggests that additional development is likely to increase the likelihood and severity of disease outbreaks.

Cohabitation is a reliable method for infecting *O. angasi* with *B. exitiosa.* This facilitates a range of studies, including examining the host-parasite interaction, development and persistence of disease and/or infection. Most importantly, a controlled infection model is necessary to facilitate a program to breed lines of Native Oysters that are resistant to *B. exitiosa.* Such programs need to be directed and use characterised family lines, to achieve substantial improvement in survival in a commercially acceptable timeframe (Lynch et al., 2014). Such a program is, in the long term, the most likely way to develop a viable Australian aquaculture industry for Native Oysters without ongoing business uncertainty caused by *B. exitiosa*-related mortality.

Live oysters, which are sampled for heart smear, have higher *B. exitiosa* prevalence than dead samples, probably due to rapid decomposition creating substantial post-mortem artefacts (Howard & Smith, 1983) and haemocyte agglutination (Smith et al., 2016) reducing cell transfer to slides and decreasing sensitivity of tests. This is important in outbreaks, because dead oysters may be tested by heart smear and not show signs of infection.

6.7 Conclusion

Bonamia exitiosa is highly pathogenic in *Ostrea angasi*, and infects, proliferates and causes mortality in this host rapidly. Host death is not required for transmission and cohabitation forms a sound basis for an infection model to study *B. exitiosa* in a controlled manner, including infection dynamics, pathogenesis and developing resistant family lines.

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7. SARDI - Susceptibility of *Crassostrea gigas* to *Bonamia exitiosa*

7.1 Summary

The Pacific oyster, *Crassostrea gigas*, was assessed for susceptibility to *Bonamia exitiosa* by cohabitation. Pacific oysters are susceptible to *B. exitiosa* and were infected rapidly but did not develop clinical disease. It is unclear if *C. gigas* is a functional host, but it is clear that it should be regarded as a translocation risk when considering movement of Pacific oysters, particularly to areas where other species of oysters that are more susceptible to *B. exitiosa* infection are farmed.

7.2 Introduction

A health survey in South Australia in 2003 identified *Bonamia*-like cells in healthy *Crassostrea gigas* using histopathology, but the diagnoses could not be confirmed (Diggles, 2003). Lynch et al. (2010) recorded *Bonamia* in *C. gigas* from Spain and Ireland using polymerase chain reaction (PCR), histology and *in situ* hybridisation (ISH) but was unable to clarify if *C. gigas* was a reservoir of *Bonamia* infection and contributing to infection pressure in *Bonamia* affected oyster growing areas. In Australia, *Ostrea angasi* is often cultured in proximity to aquaculture or naturalised populations of *C. gigas*. Determining infection status and dynamics of *B. exitiosa* infection in *C. gigas* is important for understanding field observations, the risk associated with translocation and sound principles for planning co-development of *O. angasi* and *C. gigas* farming.

7.3 Objectives

We aimed to examine susceptibility of Australian farmed populations *of C. gigas* to *B. exitiosa* and to obtain preliminary information on changes in *B. exitiosa* prevalence and infection intensity in *C. gigas* over time and if *B. exitiosa* infection influenced mortality of *C. gigas*.

7.4 Methods

Recipient juvenile (mean weight ± SE: 0.21 ± 0.00 g, mean shell length ± SE: 12.30 ± 0.09 mm) *C. gigas* were obtained from the South Australian Research and Development Institute (SARDI) SA Aquatic Sciences Centre (SAASC) Mollusc Hatchery (West Beach, Adelaide, South Australia). 150 juvenile *C. gigas* were sampled prior to the experiment and analysed via quantitative PCR (Corbeil et al., 2006 *Bonamia* sp. qPCR) and 150 were analysed by histology. *Bonamia exitiosa* was not detected in the experimental *C. gigas*. Adult donor *O. angasi* (mean weight ± SE: 86.07 ± 2.20 g, mean shell length ± SE: 78.35 ± 0.88 mm) were sourced from a farm in Coffin Bay with high *B. exitiosa* prevalence (Bayesian calculated prevalence, credible intervals: 0.90, 0.78–0.99) (Buss, Wiltshire, Prowse, Harris, & Deveney, 2018). All oysters were acclimated separately in 500 L tanks, with continuous aeration at the South Australian Aquatic Biosecurity Centre (SAABC), Roseworthy Campus and fed 15 L every 2–3 days a mixture of *Chaetoceros muelleri, Skeletonema costatum and Pavlova lutheri* per tank.

The experiment commenced on 18th March 2018. The experiment was conducted in four plastic tanks (52 L volume), containing seawater with continuous aeration. Every 2–3 days 100% of the seawater was exchanged and the oysters were fed 1.25 L (2.0 x 10^6 cells/mL) of a mixed culture of *Chaetoceros muelleri, Skeletonema costatum and Pavlova lutheri*. For the experiment, three tanks contained 10 donor *O. angasi* and 500 recipient *C. gigas*. The control tank contained 500 *C. gigas*. Throughout the experiment laboratory air temperature was 18°C, salinity was 38 ppt, total ammonia was 0 mg/L and dissolved oxygen was 96.67 ± 0.15% or 7.20 ± 0.03 mg/L. Water temperature was (mean ± SE) 16.41 ± 0.06°C. Exchange water was 22°C and took 5–6 h to cool down to ~16°C. Every 2–3 days oysters were checked for mortalities.

Oysters were sampled on day 20 and day 60. On each sampling day, 30 recipient oysters per tank were sampled (total n=90) and 60 control oysters were sampled for heart smear, histology and qPCR. All sampled oysters were replaced with additional live oysters immediately after they were removed from the tanks so that the biomass was the same throughout the experiment. On day 60, all *O. angasi* donor adults (n=30) were also sampled. From the samples taken, only heart smears were further analysed by light microscopy with all intensities assessed using the scoring system outlined by Buss et al. (2018).

Parasitology terminology used is consistent with Bush et al. (1997). Apparent prevalence was defined as the proportion of individuals that were positive for a single test, with the assumption that the test was perfect. Estimated prevalence was calculated from outputs of a Bayesian model informed by priors based on relevant data and provides credible intervals which permit a more statistically robust estimate of prevalence. Prevalence (apparent and estimated) was based on heart smear test results. Prevalence (apparent and estimated) was based on heart smear test results.

Estimated prevalence with credible intervals were estimated using a Bayesian JAGS code modified from the R Package “prevalence” (Devleesschauwer et al., 2015). JAGS was run using the “R2jags” package (Su & Yajima, 2015) in R (R Core Team, 2017). For this analysis, posterior predictions from DSe, DSp and conditional covariance for a positive or negative disease status calculated in Buss et al. (2018) were used to inform priors for heart smear. Within the new model, the following beta priors: (113, 72) and (27, 18) were used for heart smear DSe and DSp respectively. Overlapping credible intervals signified no difference.

Mean intensity was assessed using Quantitative Parasitology 3.0 (Reiczigel et al., 2005), using Sterne’s exact test to provide 95% bootstrap confidence intervals with 2000 replications. Overlapping confidence intervals signified no difference.

Generalised Linear Models (GLMs) with binomial distribution were used to assess changes in prevalence in heart smear data over time and between recipient and donor oysters. GLMs were also used to assess *B. exitiosa* intensity and abundance in oysters across over time and between oyster age (juveniles and adults). A negative binomial distribution was assumed for all intensity and abundance analyses, due to over dispersion of data relative to a Poisson distribution. GLMs were conducted in R using the “stats” package (R Core Team, 2017) and negative binomial GLMs used the “MASS” package (Venables & Ripley, 2002).

7.5 Results

Mortalities in recipient animals throughout the trial could not be sampled due to lack of tissue. Recipient animals displayed 0.20% mortality and control oysters displayed 0% mortality over the experiment.

Bonamia exitiosa was detected in live recipient *C. gigas* by heart smear (Figure 30). Apparent prevalence in recipient *C. gigas* was <34% over the duration of the experiment, with 96.67% for donor *O. angasi* adults (Table 25). *Bonamia exitiosa* was not detected in control *C. gigas*.

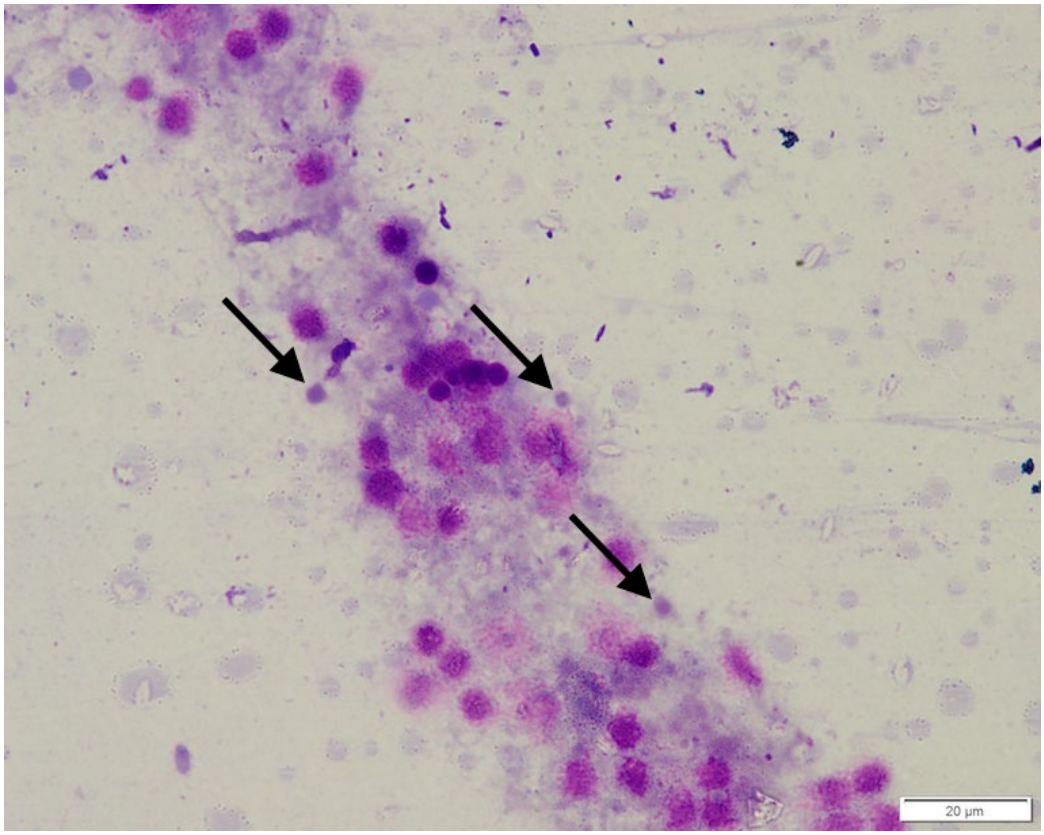

Figure 31. Heart smear stained with a Hemacolor® kit. *Bonamia exitiosa* (arrows) in *Crassostrea gigas*. Scale bar= 0 µm

There was no significant difference in apparent heart smear prevalence in juvenile recipient *C. gigas* on day 20 and day 60 (CHI: $\chi 2$ (1) =0.10, p=0.75). Adult donor *O. angasi* sampled on day 60 had significantly higher apparent heart smear prevalence than juvenile recipient *C. gigas* sampled on day 60 (CHI: $\chi 2$ (1) =45.79 p=1.32e-11) (Figure 32). Estimated prevalence was higher in donor *O. angasi* (mean, credible intervals: 0.96, 0.86–1.00) than in recipient *C. gigas* (<0.35, 0.21–0.51) (Table 25). Recipient *C. gigas* had higher estimated prevalence than control *C. gigas* and there was no change in estimated prevalence over time (Table 25).

Table 25. Size data, apparent prevalence, Bayesian estimated prevalence with credible intervals (calculations used known DSe, DSp and covariance values for heart smear as priors) and mean intensity with confidence intervals (calculated through Quantitative Parasitology) of *Bonamia exitiosa* in *Crassostrea gigas* juveniles and *Ostrea angasi* adult oysters from heart smears at days 20 and 60 for control and exposed treatments. Confidence intervals could not be calculated when values were constant. Intensity cell counts were capped to 30 cells maximum

			Size data					Bayesian	Quantitative Parasitology (bootstrap, 95%)
Treatment sampling day	Treatment	Species	Weight (g) (mean±SE)	Shell length (mm) (mean±SE)	Meat:shell ratio (%) (mean±SE)	n	Apparent prevalence (%)	Estimated prevalence (credible intervals)	Mean heart smear intensity cell count (confidence intervals)
20	Exposed	*C. gigas*	0.22±0.01	12.94±0.19	97.12±2.42	90	33.33	0.35 (0.21–0.51)[b]	2.33 (1.90–2.93)[b]
60	Exposed	*C. gigas*	0.24±0.01	12.37±0.13	71.21±2.42	90	31.11	0.32 (0.18–0.47)[b]	7.07 (5.07–10.60)[a]
20	Control	*C. gigas*	0.16±0.01	12.04±0.20	89.94±3.20	60	0	0.02 (0.00–0.08)[c]	0
60	Control	*C. gigas*	0.19±0.01	11.49±0.14	78.73±3.92	60	0	0.02 (0.00–0.08)[c]	0
60	Exposed	*O. angasi*	85.08±3.10	77.64±1.30	30.23±0.89	30	96.67	0.96 (0.86–1.00)[a]	5.93 (4.59–7.48)[a]

† Different superscripts denote differences at a 5% level, with [a] representing the highest value.

‡ Control and exposed treatments included live *C. gigas* juveniles sampled on days 20 and 60. The adult treatment included live *O. angasi* adults sampled on day 60.

§ Priors for the heart smear were derived from Buss et al. (2018). SE: standard error; n: sample number per treatment.

Intensity in recipient *C. gigas* was low (mean, confidence interval: between 2.33, 1.90–2.93 and 7.07, 5.07–10.60 cells) and scored only as a light infection (Buss et al., 2018) (Table 25). Recipient *C. gigas* had significantly higher heart smear intensities at day 60 than those sampled at day 20 (CHI: $\chi 2$ (1) =27.77 p=1.37e-7) (Figure 33). *Bonamia exitiosa* intensity increased over time for recipient *C. gigas*, with higher intensity at day 60 (mean, confidence intervals: 7.07, 5.07–-10.60) than at day 20 (2.33, 1.90–2.93) and higher intensity than control oysters (remained 0) at both sample times (Table 25). There was no significant difference in heart smear intensity between recipient *C. gigas* and donor *O. angasi* at day 60 (CHI: $\chi 2$ (1) =0.91 p=0.34) (Figure 34).

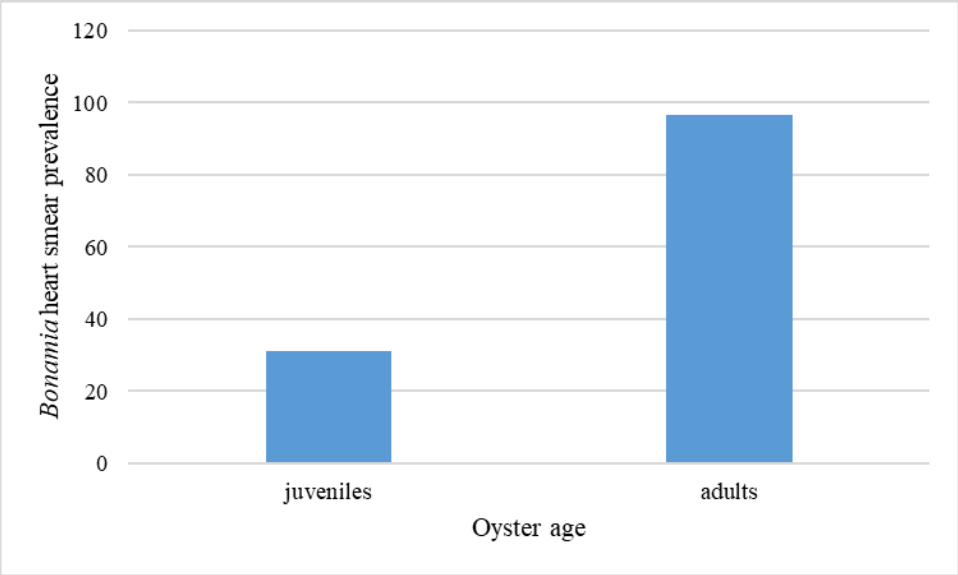

Figure 32. Apparent Bonamia sp. heart smear prevalence in live recipient *Crassostrea gigas* and donor *Ostrea angasi* sampled on day 60

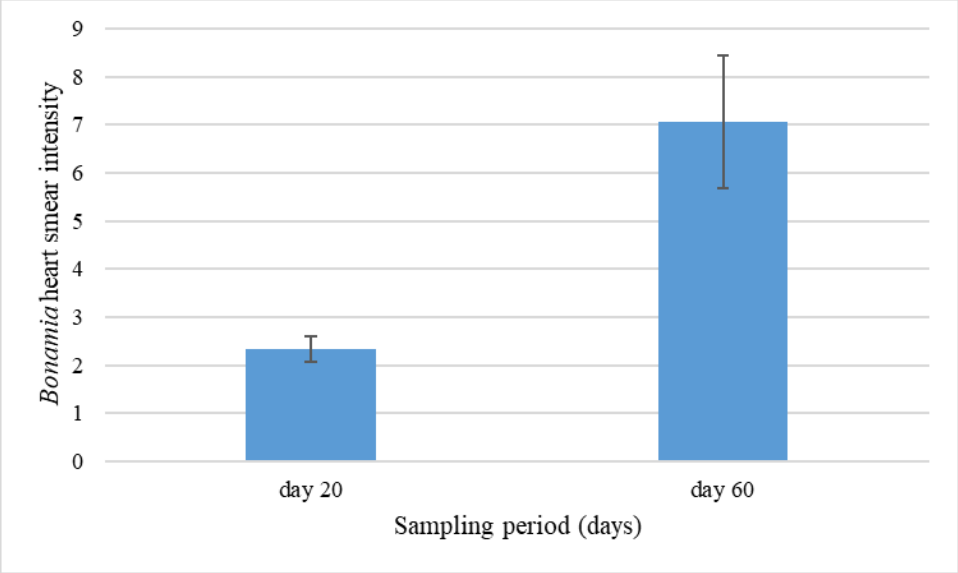

Figure 33. Bonamia sp. intensity from heart smears in recipient *Crassostrea gigas* sampled on days 20 and 60. Mean±SE

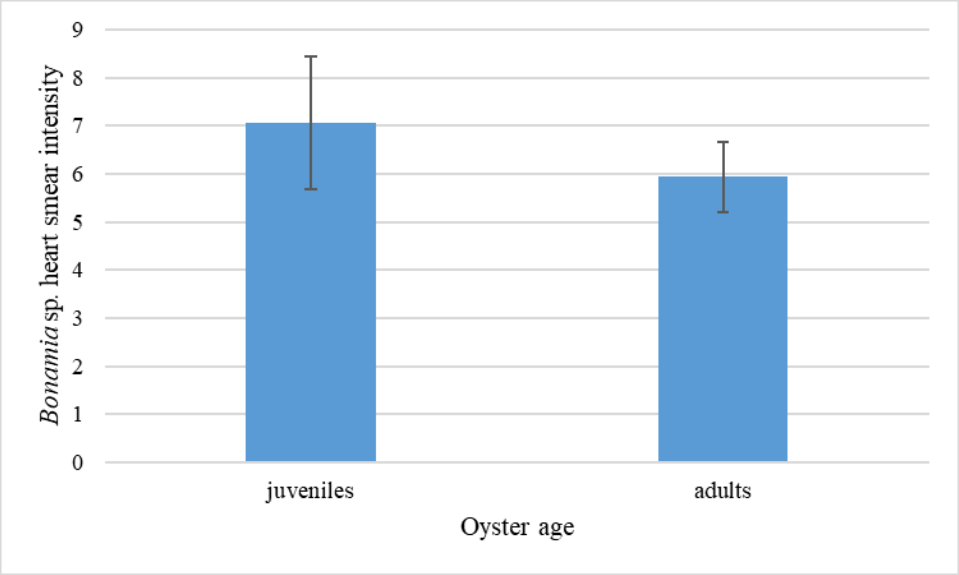

Figure 34. Bonamia sp. intensity from heart smears for recipient *Crassostrea gigas* and donor *Ostrea angasi* adults sampled on day 60. Mean±SE

Recipient *C. gigas* sampled on day 60 had significantly higher heart smear abundance than recipient *C. gigas* sampled on day 20 (CHI: $\chi 2$ (1) = 7.69 p=5.57e-3) (Figure 35). Donor *O. angasi* sampled on day 60 had significantly higher heart smear abundance than recipient *C. gigas* sampled on day 60 (CHI: $\chi 2$ (1) = 7.59 p=5.85e-3) (Figure 36).

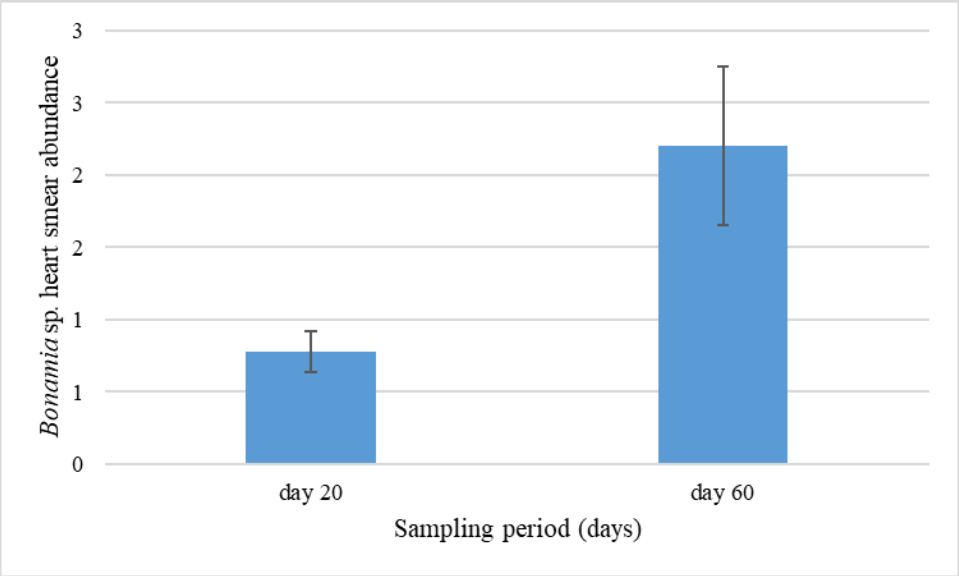

Figure 35. *Bonamia sp.* abundance from heart smears for recipient *Crassostrea gigas* sampled on days 20 and 60. Mean ± SE

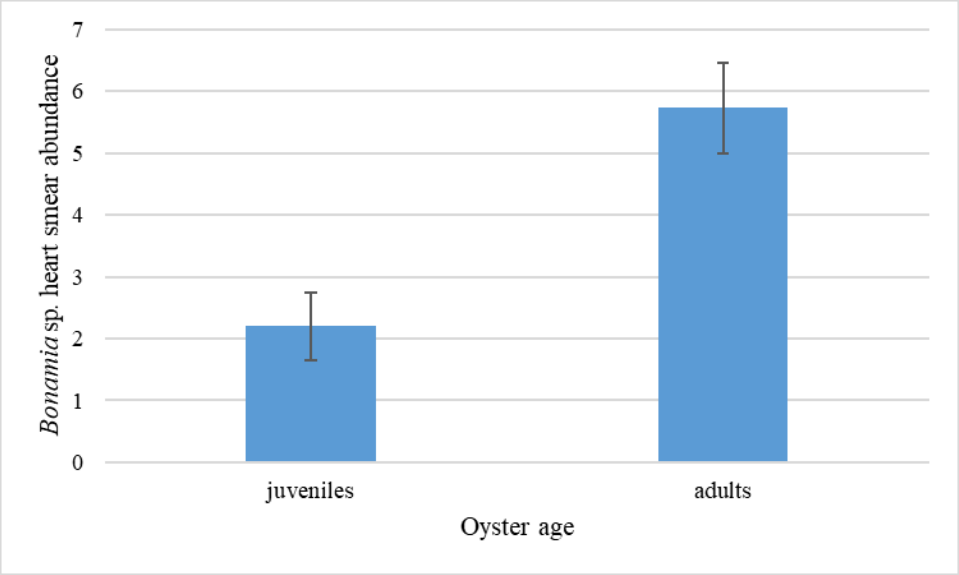

Figure 36. *Bonamia sp.* abundance from heart smears for recipient *Crassostrea gigas* and donor *Ostrea angasi* sampled on day 60. Mean ± SE

7.6 Discussion

As suggested by Lynch et al. (2010), *C. gigas* are susceptible to *Bonamia*. Our results demonstrate rapid *B. exitiosa* infection; by day 20 estimated prevalence was 0.35, although prevalence did not increase over the trial. Infection intensity, however, was low compared to intensity of *B. ostreae* in *O. edulis* in European farms (Culloty et al., 2004) or those we observed in recipient *O. angasi* after a similar time in our *O. angasi* cohabitation trial (mean, confidence intervals: 16.20, 12.70–20.70 (Table 24, section 6).

The lack of observed mortality indicates that *B. exitiosa* is unlikely to cause disease in *C. gigas*. Over a comparable period, juvenile *O. angasi* began to die by day 12 and suffered up to 87% mortality by day 40 (Section 6, Figure 20). Given the importance of immune system differences between individual *O. edulis* for *Bonamia* resistance (Gervais et al., 2016) and the variety of mechanisms of resistance (Morga et al., 2012) it suggests that *C. gigas* has different cellular immune defences that prevent the development of clinical disease (Gervais et al., 2016).

It is relevant to all industries that farm *C. gigas* and other oysters that are more susceptible to disease caused by *Bonamia* spp. that co-culture and translocations pose a likely risk to the sustainability of farming the susceptible ostreine oysters, *O. angasi* particularly in Australia. Careful spatial planning is essential when farming *C. gigas* and *O. angasi* or other oysters that are susceptible to disease caused by *Bonamia* spp.

Donor animals do not need to have high *Bonamia* intensity for transmission to occur to recipient juveniles; donor oyster intensity was mean, confidence intervals 5.93, 4.59–7.48 at day 60 (Table 25). Donor animals did have high prevalence mean, credible intervals: 0.96, 0.86–1.00 (Table 25). It is possible that prevalence has a greater influence on transmission than intensity for *B. exitiosa* transmission.

Further experiments should assess if other cultured oysters such as *Saccostrea* spp. are susceptible to *B. exitiosa* and the effects of long term holding on *B. exitiosa* prevalence and intensity in *C. gigas* and other oyster species. It is important for oyster farming sustainability to determine if *C. gigas* is a functional host for *B. exitiosa* including if it can transmit *B. exitiosa* to more susceptible hosts such as *O. angasi*. Populations of *C. gigas* in proximity to *O. angasi* farms should be surveyed to assess changes in *B. exitiosa* prevalence and intensity, seasonality and to better quantify the biosecurity threat posed by farming *C. gigas* with *O. angasi*.

7.7 Conclusion

Pacific oysters are susceptible to *B. exitiosa* by cohabitation and Pacific oysters should be regarded as a translocation risk when considering movement of Pacific oysters, particularly to areas where other species of oysters are farmed that are more susceptible to *B. exitiosa* infection.

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8.SARDI - *Bonamia exitiosa* farm trial

8.1 Summary

We deployed uninfected juvenile *Ostrea angasi* on commercial oyster farms at Cowell, two sites in Coffin Bay and at Streaky Bay in South Australia, in areas where mostly Pacific oysters, but also some *O. angasi* are farmed. Oysters became infected rapidly with *Bonamia exitiosa*, with typical, mostly low abundance, infections. Oysters at Cowell had the lowest *B. exitiosa* prevalence and grew more than twice as quickly as oysters at other sites, despite high temperature variation and high salinity. Oysters at Coffin Bay had the highest *B. exitiosa* prevalence, with some oysters showing infections that could reflect clinically significant infections. None of the environmental parameters we measured were correlated with infection intensity or prevalence, and the environmental drivers of *B. exitiosa* infections in South Australia remain unclear. Cowell and Streaky Bay are sites that may provide a better environment for development of an industry to grow *O. angasi*.

8.2 Introduction

Following the discovery of *B. exitiosa* in South Australian Native Oyster farms, we undertook a field trial on *O. angasi* commercial farms in three growing areas in South Australia encompassing a range of environments.

8.3 Objectives

We aimed to assess survival, seasonal dynamics of *B. exitiosa* infection and if environmental variables could be related to the prevalence and intensity of *B. exitiosa* infection. Spat were deployed at Franklin Harbour (Cowell), Coffin bay (two sites) and Streaky Bay in January–February 2017 and monitored for 12–13 months.

8.4 Methods

Oysters were deployed on four oyster farms operated by commercial growers who volunteered to be involved in the project. Coffin Bay B is a site in the Deadman's group of farms in Western Coffin Bay. It is a relatively low energy environment that is shallow and dry at low tide, sheltered from prevailing winds, with a sandy seafloor and high biofouling mostly comprising barnacles. Coffin Bay Z is a site in the Central group of farms in the middle main channel part of Coffin Bay. It is a high energy site with deep water and has a large tidal fluctuation; the farm is intertidal but in the middle of the tidal range, with a sandy substrate and high biofouling comprising barnacles and mussels. The Cowell site is a farm in southern Franklin Harbour. It is a low energy site with shallow water that is dry at low tide, adjacent a mangrove forest, with a fine sediment substrate and dense *Posidonia* sp. beds. The Cowell site has high turbidity and extreme barnacle biofouling, particularly in summer. The Streaky Bay site is a shallow site with high wave and wind exposure. This site is characterised by a sandy sea floor with *Posidonia* sp. beds and a large razor fish (*Pinna bicolor*) population.

Prior to deployment, hatchery stock were tested by real-time PCR and histology and found no *B. exitiosa* (mean Bayesian calculated prevalence, credible intervals: 0.017, 0.000–0.05: Buss, Wiltshire, Prowse, Harris, & Deveney, 2018). Post testing, 500 g of 7–10 mm *O. angasi* spat

were transferred to each site. Spat were held under commercial conditions, in three baskets (SEAPA, Edwardstown, SA) per site; 3 mm mesh baskets were used at Coffin Bay and Cowell and 6 mm SEAPA baskets were used in Streaky Bay. Stocking and sampling times are shown in Table 26. A conductivity probe (Odyssey Conductivity and Temperature Loggers; 80 mS/cm) logging every 12 h was placed at the base of the post holding the experimental oysters. Two temperature loggers (HOBO Pendant® Temperature/Light Data Logger 64K-UA-002-64) were deployed; one on an oyster post at the same level as the oysters, one at the base of the post, both logging every 15 min. The above temperature probe represented the temperatures the oysters were experiencing.

Table 26. Sampling timeline of *Ostrea angasi* from four South Australian sites (Coffin Bay B, Coffin Bay Z, Cowell and Streaky Bay) over four seasons†

YEAR	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
	S	S	A	A	A	W	W	W	Sp	Sp	Sp	S
2017		St			X1			X2			X3	
2018		X4										

†Where 'St' denotes date of stocking, 'X' denotes sampling date and the number corresponding to the number of sampling trips since stocking. 'S' is summer, 'A' is Autumn, 'W' is winter and 'Sp' is spring.

Oysters were sampled seasonally from May 2017 (Table 26). At the end of each season 60 oysters were randomly collected per site (20 per basket). Each oyster was sampled for heart smear, histology and qPCR. Each oyster was weighed, measured and the meat:shell ratio was calculated. Because confirmation of *B exitiosa* infection in South Australian farmed *O. angasi* populations has already been achieved using qPCR, histology and heart smear (Buss, et al., 2018) only heart smears were analysed further from this study as they provide information on changing prevalence, intensity and are cost effective.

Diagnostics were undertaken as outlined above. Heart smears were scored for intensity using the grading system in Buss et al. (2018).

Parasitology terminology is used consistent with Bush, Lafferty, Lotz & Shostak (1997). Apparent prevalence was defined as the proportion of individuals that were positive for a single test, with the assumption that the test was perfect. Estimated prevalence was calculated from outputs of a Bayesian model informed by priors based on relevant data and provides credible intervals which permit a more statistically robust estimate of prevalence. Prevalence (apparent and estimated) was based on heart smear test results.

Estimated prevalence with credible intervals were estimated using a Bayesian JAGS code modified from the R Package "prevalence" (Devleesschauwer et al., 2015). JAGS was run using the "R2jags" package (Su & Yajima, 2015) in R (R Core Team, 2017). For this analysis, posterior predictions from DSe, DSp and conditional covariance for a positive or negative disease status calculated, as described by Buss et al. (2018), were used to inform priors for heart smear. Within the new model, the following beta priors: (113, 72) and (27, 18) were used for heart smear DSe and DSp respectively. Overlapping credible intervals signified no difference.

Intensity data were analysed using the software Quantitative Parasitology 3.0 (Reiczigel, Zakariás & Rózsa, 2005), using Sterne's exact confidence intervals for 95% bootstrap

confidence intervals for mean intensities (with 2000 replications). Overlapping confidence intervals, signified no difference.

Generalised Linear Models (GLM) with binomial distribution were used to assess apparent prevalence for oysters across site and season. GLMs were also used to assess *B. exitiosa* intensity and abundance in oysters across site and season. A negative binomial distribution was assumed for all intensity and abundance analyses, due to over dispersion of data relative to a Poisson distribution. GLMs were conducted in R using the "stats" package (R Core Team, 2017) and negative binomial GLMs used the "MASS" package (Venables & Ripley, 2002).

To assess the effect of environmental variables, a scatterplot matrix was created using the "lattice" package in R (Sarkar, 2018).

8.5 Results

Bonamia exitiosa was detected at all sites by the end of the first season in autumn (mean, credible intervals: >0.07, 0.00–0.25) (Table 27). From autumn to winter, there was a rapid increase in estimated prevalence over time at all sites (mean, credible intervals: >0.44, 0.05–0.86). After winter, estimated prevalence across sites remained high (>0.50) for the rest of the year (Table 27). Cowell had the lowest estimated prevalence at the end of the trial 0.56 (0.13–0.94), while all other sites had estimated prevalence's >0.78 (Table 27). Oysters from both Coffin Bay sites had significantly higher apparent *B. exitiosa* prevalence than oysters from Cowell and Streaky Bay and Streaky Bay had significantly higher *B. exitiosa* prevalence than Cowell (CHI: $\chi 2$ (3) =21.99, p=6.54e-5) (Figure 37). Apparent *B. exitiosa* prevalence increased significantly over time, with oysters sampled in autumn having significantly lower apparent *B. exitiosa* prevalence than other sampling periods. Oysters sampled in summer had highest apparent *B. exitiosa* prevalence (CHI: $\chi 2$ (3) =45.03, p=9.10e-10) (Figure 38).

Table 27. Size data (mean ± standard error), apparent prevalence (%), Bayesian estimated prevalence (%) and mean intensity (calculated through Quantitative Parasitology) of *Bonamia exitiosa* in *Ostrea angasi* from heart smears over autumn, winter, spring and summer at Coffin Bay B, Coffin Bay Z, Cowell and Streaky Bay in South Australia[†‡]

		Size data				Bayesian		Quantitative parasitology (bootstrap 95%)
	Site	Weight (g) (Mean ± se)	Shell length (mm) (Mean ± se)	Meat:shell ratio (%) (Mean ± se)	n	Apparent prevalence (%)	Estimated prevalence (credible intervals)	Mean heart smear intensity cell count (confidence intervals)
Autumn	Coffin Bay B	6.02±0.26	39.77±0.62	45.20±0.18	60	43.33	0.40 (0.06–0.78)^{ab}	3.65 (2.65–4.96)^{b}
	Coffin Bay Z	6.66±0.27	38.91±0.55	38.63±0.21	60	40.00	0.32 (0.03–0.68)^{ab}	4.38 (2.83–7.8)^{ab}
	Cowell	10.17±0.83	45.31±1.75	46.10±0.61	60	23.33	0.09 (0.00–0.30)^{b}	3 (1.79–5)^{b}
	Streaky Bay	3.05±0.15	29.70±0.53	44.65±0.11	60	20.00	0.07 (0.00–0.25)^{b}	4 (2.5–7.26)^{ab}
Winter	Coffin Bay B	8.99±0.74	42.88±1.04	43.49±0.52	40	52.50	0.61 (0.19–0.96)^{ab}	3.86 (3–5)^{b}
	Coffin Bay Z	5.30±0.36	33.01±0.74	37.72±0.26	59	67.80	0.88 (0.62–1.00)^{a}	7.02 (5.25–9.52)^{a}
	Cowell	9.44±0.70	44.04±1.10	47.64±0.54	40	35.00	0.50 (0.08–0.91)^{ab}	3.5 (2.43–5.07)^{b}
	Streaky Bay	3.70±0.22	28.56±0.62	39.86±0.16	40	45.00	0.44 (0.05–0.86)^{ab}	3.89 (2.61–5.64)^{ab}
Spring	Coffin Bay B	6.72±0.47	38.95±0.86	40.41±0.34	40	72.50	0.88 (0.61–1.00)^{a}	4.55 (3.17–8.01)^{ab}
	Coffin Bay Z	5.89±0.64	32.61±1.04	35.80±0.48	40	60.00	0.76 (0.37–0.99)^{a}	4.29 (3.33–5.33)^{ab}
	Cowell	10.11±0.97	46.46±1.56	50.93±0.66	40	47.50	0.50 (0.10–0.90)^{ab}	3.21 (2.21–4.42)^{b}
	Streaky Bay	5.88±0.45	35.11±1.21	38.67±0.33	40	55.00	0.67 (0.26–0.97)^{ab}	2.86 (2.05–4)^{b}
Summer	Coffin Bay B	7.28±0.74	36.96±0.97	35.83±0.54	40	62.50	0.78 (0.42–0.99)^{a}	3.56 (2.72–4.88)^{b}
	Coffin Bay Z	8.31±0.65	36.32±0.83	29.68±0.49	40	65.00	0.81 (0.48–0.99)^{a}	5.5 (4–9.48)^{ab}
	Cowell	25.25±1.53	58.94±1.20	38.28±1.10	40	50.00	0.56 (0.13–0.94)^{ab}	3.7 (2.9–4.75)^{b}
	Streaky Bay	9.51±0.56	43.04±0.76	42.34±0.41	40	62.50	0.79 (0.43–0.99)^{a}	3 (2.4–4)^{b}

† Different superscripts denote differences at a 5% level, with ^{a} representing the highest value.

‡ se: standard error; n: sample number per site.

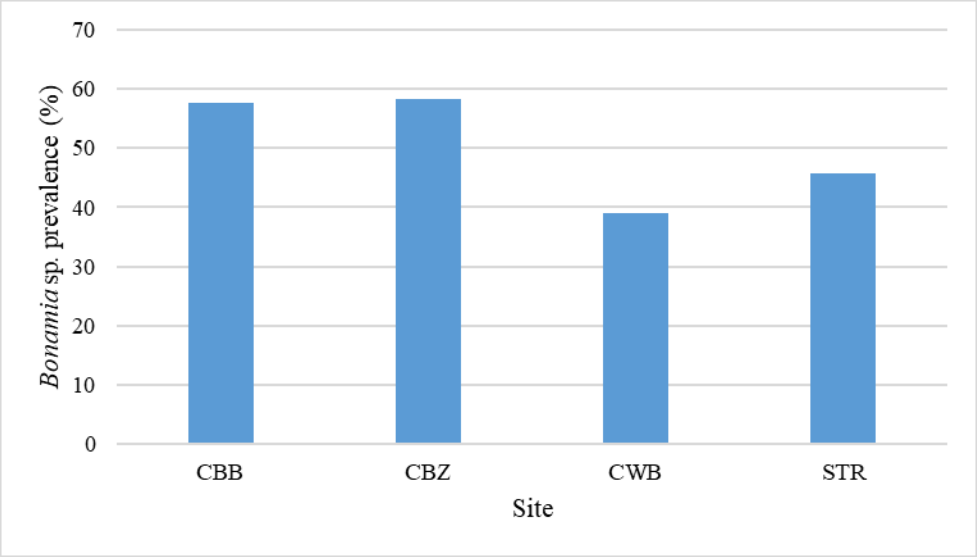

Figure 37. Apparent Bonamia sp. prevalence in Ostrea angasi sampled at four sites (Coffin Bay B: CBB, Coffin Bay Z: CBZ, Cowell: CWB, Streaky Bay: STR) over four seasons per site (autumn, winter, spring and summer)

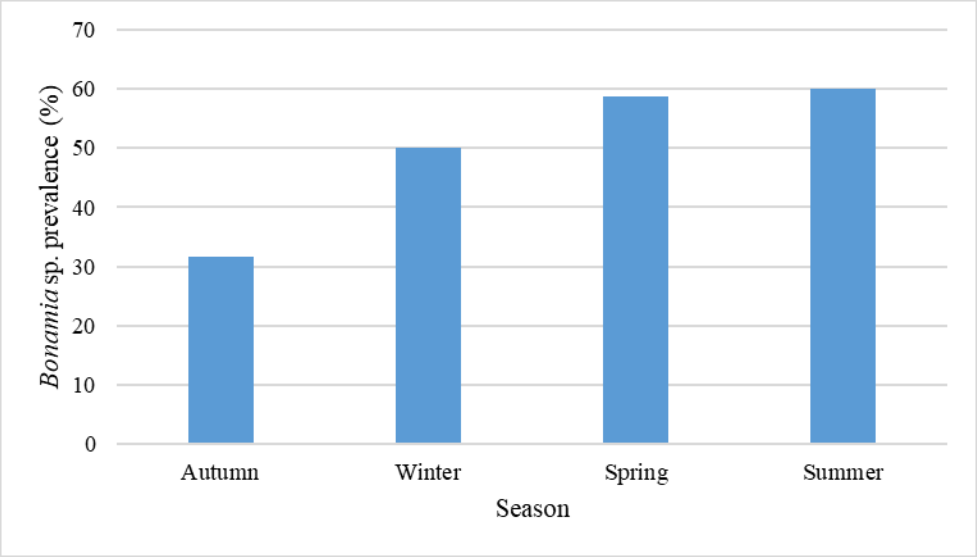

Figure 38. Apparent *Bonamia sp.* prevalence in *Ostrea angasi* sampled over four seasons (autumn, winter, spring and summer) at all sites (Coffin Bay B, Coffin Bay Z, Cowell and Streaky Bay)

Intensity was classed as light infection (Buss et al., 2018) throughout the year at all sites (mean, confidence intervals: <7.02, 5.25–9.52, Table 27). Oysters from Coffin Bay Z had significantly higher *B. exitiosa* intensity than oysters from other sites. There was no significant difference in *B. exitiosa* intensity in oysters from Coffin Bay B, Cowell and Streaky Bay (CHI: $\chi 2$ (3) =27.57, p=4.47e-6) (Figure 39). The highest *B. exitiosa* intensity was observed in oysters collected in winter from Coffin Bay Z (Table 27).

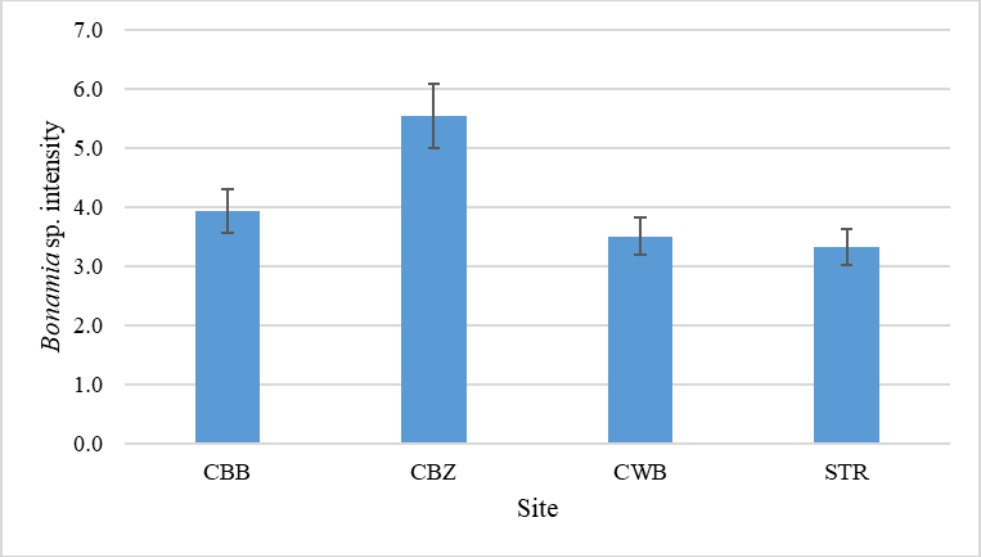

Figure 39. *Bonamia sp.* intensity in *Ostrea angasi* sampled at all sites (Coffin Bay B: CBB, Coffin Bay Z: CBZ, Cowell: CWB, Streaky Bay: STR) in all seasons. Mean ± SE

Oysters from Coffin Bay Z had significantly higher *B. exitiosa* abundance than oysters sampled from other sites. Oysters from Coffin Bay B had significantly higher abundance than oysters sampled from Cowell or Streaky Bay sites. There was no significant difference in *B. exitiosa* abundance in oysters from Cowell and Streaky Bay (CHI: $\chi 2$ (3) =35.82, p=8.19e-8) (Figure 40). Oysters sampled in autumn had significantly lower *B. exitiosa* abundance than oysters sampled in all other seasons. There was no significant difference in *B exitiosa* abundance in oysters sampled in winter, spring and summer (CHI: $\chi 2$ (3) =21.85, p=7.01e- 5) (Figure 41).

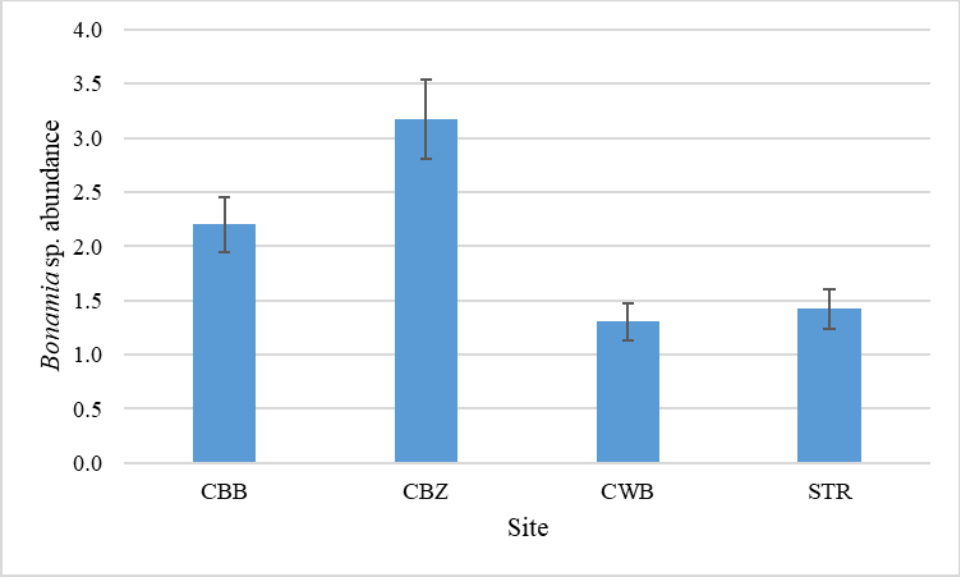

Figure 40. *Bonamia sp.* abundance in *Ostrea angasi* sampled at Coffin Bay B: CBB, Coffin Bay Z: CBZ, Cowell: CWB, Streaky Bay: STR in all seasons. Mean ± SE

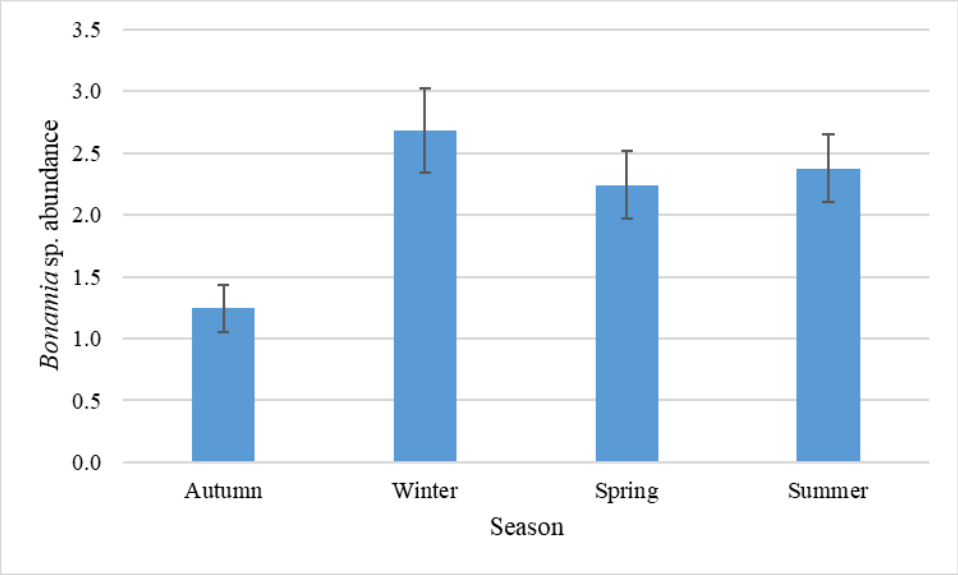

Figure 41. Seasonal *Bonamia sp.* abundance in *Ostrea angasi* all sites. Mean ± SE

Oyster survival was similar at the end of the trial between both Coffin Bay sites and Cowell (21.69– 32.77%) (Table 28). Streaky Bay had the lowest survival at the end of the trial (17.01%) (Table 28). This was likely due to larger sized baskets being used (6 mm, not 3 mm) at this site. A substantial proportion of the oysters were probably lost during a heavy weather event at the start of the trial and the 6 mm mesh may not have been adequate to contain the oysters.

Table 28. Survival of Ostrea angasi from South Australia oyster farms after one year deployed in farms

	Count at stocking	Final total counts (dead and alive) †	Final counts (live) †	Final live count including oysters removed for sampling in 1st 3 seasons (180) †	Oyster survival, end of trial (%)‡
Coffin Bay B	1785	685	405	585	32.77
Coffin Bay Z	1785	399	206	386	21.62
Cowell	1785	438	234	414	23.19
Streaky Bay	1775	181	122	302	17.01

‡ Proportion live oysters includes the number of oysters sampled throughout the year

Oysters from Cowell had higher mean weight than oysters from other sites in every season. By the summer sampling period, the mean weight of oysters from Cowell (mean: 25.25 g) were more than double the mass of oysters from other sites (7.28–9.51 g) (Table 27).

Cowell had higher water conductivity than other sites (Table 29). Cowell also had higher temperatures at oyster level in autumn, spring and summer than other sites, and was colder in winter (Table 29).

Table 29. Mean seasonal water conductivity and temperature data for Coffin Bay B, Coffin Bay Z, Cowell and Streaky Bay

	Water conductivity (mS/cm) (mean±SE)†§				Water temperature: above probe (°C) (mean±SE)†				Water temperature: below probe (°C) (mean±SE)†			
	Coffin Bay B	Coffin Bay Z	Cowell	Streaky Bay	Coffin Bay B	Coffin Bay Z	Cowell	Streaky Bay	Coffin Bay B	Coffin Bay Z	Cowell	Streaky Bay
Autumn	54.03±0.16	51.04±0.52	55.65±0.11	54.53±0.16	18.63±0.03	18.75±0.03	19.34±0.04	19.19±0.04	20.77±0.03	18.67±0.02	19.28±0.03	19.34±0.03
Winter	58.49±0.09	52.44±0.79	55.13±0.20	43.01±0.91	13.18±0.01	13.53±0.07	12.82±0.01	13.49±0.01	13.20±0.01	13.35±0.01	12.76±0.01	13.53±0.01
Spring	48.81±0.54	48.06±0.65	60.33±0.16	42.48±0.71	17.68±0.21	17.34±0.04	17.88±0.04	15.74±0.07	17.28±0.03	17.07±0.03	17.98±0.03	18.23±0.03
Summer	42.84±1.40	59.14±0.26	63.13±0.15	44.23±2.30	21.64±0.07	22.74±0.02	24.21±0.04	23.73±0.03	22.69±0.02	22.64±0.02	23.58±0.02	23.29±0.02

‡ Data were collected at the end of each season.

§36 ppt at 25°C is approximately 54.4 mS/cm

Total plankton cell count was higher for Streaky Bay (mean ± SE: 856,700 ± 156,331.56 cells/L) and Coffin Bay (820,428 ± 406,718.86 cells/L), in summer compared to other sites and seasons (Figure 42), but the correlation matrix showed that neither prevalence or intensity were influenced significantly by any of the environmental variables we monitored (Figure 43).

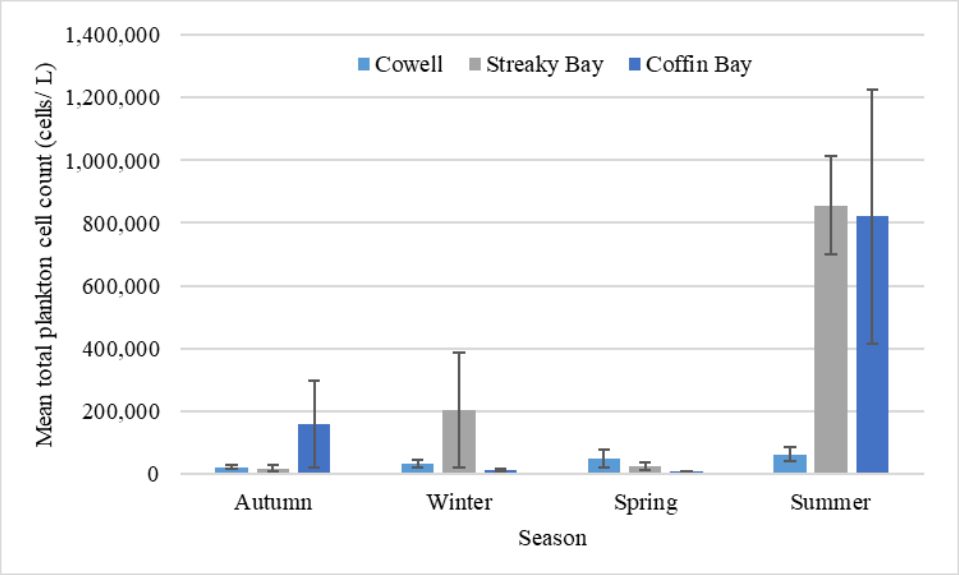

Figure 42. Total plankton cell counts (cells/L) per site (Cowell, Streaky Bay and Coffin Bay) over four seasons. Mean ± SE

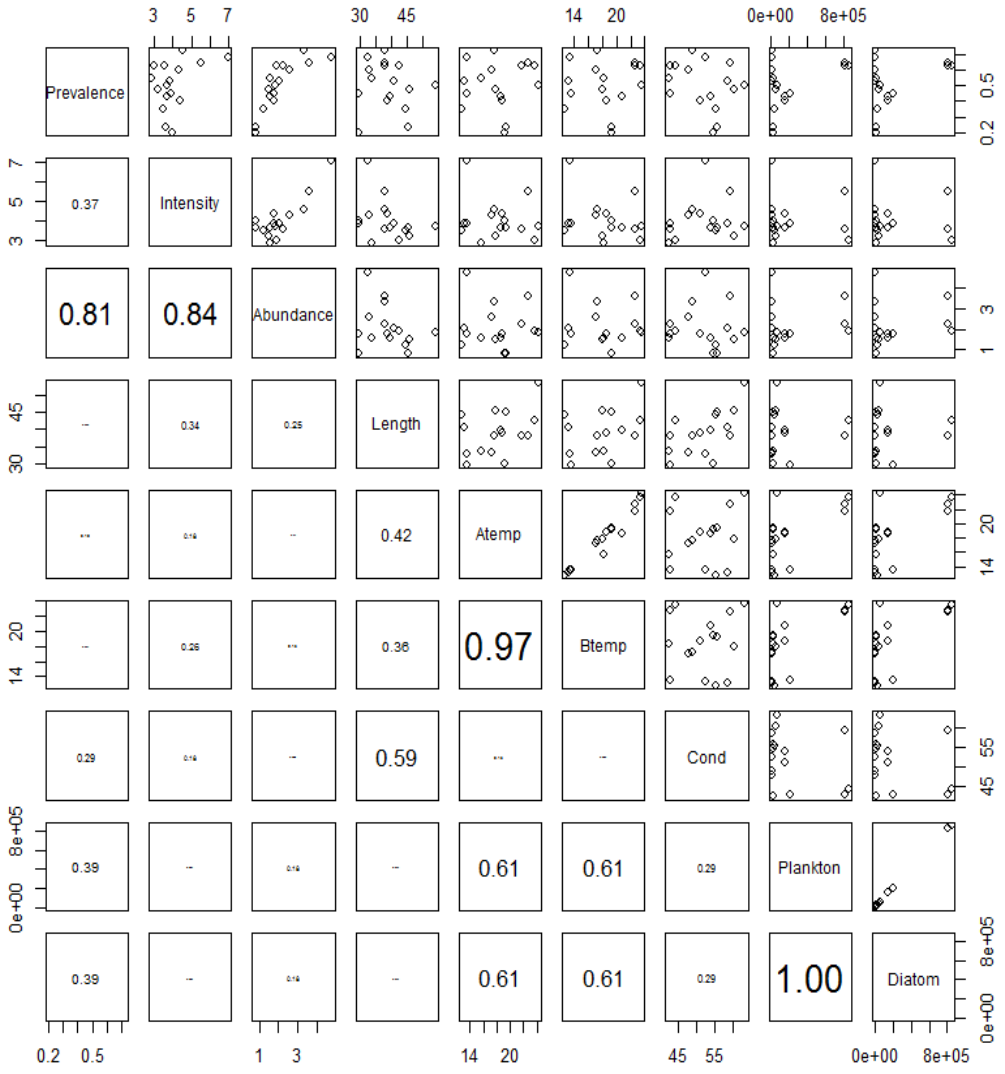

Figure 43. Correlation scatterplot matrix of *Bonamia exitiosa* prevalence, intensity, abundance; oyster length, above probe temperature (Atemp), below probe temperature (Btemp), conductivity (Cond), plankton and diatom counts. Numbers within squares are correlation coefficients

8.6 Discussion

At all sites Native Oysters were infected with *B. exitiosa* by the end of the first deployment. This is consistent with the rapid infection we observed in our laboratory trial (Section 6) and with Audemard et al., (2008a) who found that *B. exitiosa* rapidly infected *C. ariakensis* in the field. Oysters in Cowell experienced a warmer autumn, spring and summer, but a colder winter than oysters were exposed to at other sites (Table 29). The low *B exitiosa* prevalence at Cowell and the growth we observed there may be linked. These occurred despite Cowell having the highest water conductivity and the greatest seasonal temperature range. Disease caused by *B. ostreae* in *O. edulis* (see Engelsma et al., 2010), *B. exitiosa* in *O. chilensis* (see Hine et al., 2002) and by *Bonamia* sp. in *Crassostrea ariakensis* (see Audemard et al., 2008a; Audemard et al., 2008b) is more severe at higher salinity. Seasonal and temperature relationships in *Bonamia* infections, however, vary depending on the host-parasite system. *Bonamia exitiosa* had higher prevalence in *C. ariakensis* in warmer water, with clear seasonal peaks in spring, summer and early autumn with associated mortality than occurred in winter

and late autumn (Carnegie et al., 2008). *Bonamia ostreae* in *O. edulis* does not have a clear seasonal cycle but higher prevalence is reported in spring and winter (Culloty & Mulcahy, 1996; Engelsma et al., 2010). *Bonamia exitiosa* in *O. chilensis* had two seasonal prevalence peaks in winter and autumn with lowest prevalence in spring (Hine, 1991). We did not identify a clear seasonal cycle for *B. exitiosa* infection in *O. angasi*, however, increasing *B. exitiosa* prevalence over time highlights this pathogen as a serious limitation for further expansion in the current *O. angasi* industry.

There was no support for any environmental parameters, we measured, influencing prevalence or intensity of *B. exitiosa* (Figure 43). It is likely that there are factors influencing *B. exitiosa* infection dynamics that we did not measure. Oyster density is likely to be important in infection dynamics, and we have shown that *C. gigas* is likely to be a host for *B. exitiosa.* Cowell has a smaller area leased for oysters farming (115.68 ha) than Coffin Bay (165.25 ha), but higher than Streaky Bay (95 ha) (PIRSA, 2017). Leased farming area may not, however relate to number of oysters particularly given that the South Australian oyster farming industry has experienced a severe shortage of spat following the introduction in February 2016 of measures designed to prevent entry of OsHV-1 microvariant (the viral agent that causes Pacific oyster mortality syndrome) from Tasmania. Lower density and lower total biomass of farmed oysters, including *C. gigas*, could explain the lower *Bonamia* prevalence observed at Streaky Bay and Cowell, compared to Coffin Bay. Coffin Bay is an icon site for oyster farming in South Australia and due to its market position and favourable oyster growth, probably has the highest oyster biomass and number of individuals of our study sites. More information on the oyster biomass in each growing region is needed to investigate this further.

Bonamia exitiosa prevalence in farms increases over time but all intensities were classed as light infection following Buss et al. (2018). High prevalence and low intensity infections appear to be a characteristic of South Australian (Buss et al., 2018) and Australian (Corbeil et al., 2009) *B. exitiosa* infections. Higher prevalence, intensity and abundance were found in *O. angasi* in Coffin Bay than Streaky Bay or Cowell. Coffin Bay Z had the highest prevalence, intensity and abundance. Variation of prevalence and intensity between different sites has also been described for *B. ostreae* in *O. edulis* (see Culloty et al., 2004). When seven different oyster strains (from naïve or exposed oyster populations) were exposed to three different sites (Ireland, France and Holland), where *B. ostreae* is enzootic, all oyster strains were infected within three months at Cork Harbour (Ireland) and Brittany (France), whereas oysters deployed in Lake Grevelingen (Holland) appeared to be infected first at six months. Intensities also differed between site, with more heavy infections found at Cork Harbour than at the other sites (Culloty et al., 2004). This shows the importance of analysing site differences in terms of *Bonamia* prevalence and intensity to identify suitable farming locations and aid future industry management decisions. In addition, Culloty et al. (2004) showed that oysters selectively bred at Rossmore for resistance to *B. ostreae* had significantly improved tolerance to infection, as reflected in lower prevalence and intensity, and with very low mortality. Industry should seriously consider development of an *O. angasi* breeding program to develop a Native Oyster industry.

Cowell and Streaky Bay may be more suitable sites for farming *O. angasi* than Coffin Bay due to lower *B. exitiosa* prevalence and intensities. In particular, Cowell appears suitable because of substantially better growth, and it could be worth investigating the relationship between low *B*. *exitiosa* prevalence and intensity and better oyster growth at Cowell.

8.7 Conclusions

Oysters at Cowell had the lowest *B. exitiosa* prevalence and grew more than twice as quickly as oysters at other sites and may be suitable for development of an industry to grow *Ostrea angasi*. Streaky Bay may also be a suitable site for growing *O. angasi*. None of the environmental parameters we measured were correlated with infection intensity or prevalence.

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9. SARDI - Decontamination of *Bonamia exitiosa*

9.1 Summary

Decontamination is a necessary process for managing infrastructure and premises in aquaculture. Processes for decontaminating equipment that may have been exposed to *Bonamia exitiosa* were investigated. We found that quaternary ammonium detergent was not suitable for decontaminating equipment exposed to *B. exitiosa* but was suitable for cleaning. Hypochlorite however, at 4% active chlorine for 10 minutes, reliably inactivated 100% of *B. exitiosa* cells, and provides a useful basis for cleaning equipment.

9.2 Introduction

Decontamination involves a combination of physical and chemical procedures to remove fouling and inactivate target pathogens. Effective decontamination is vital as general farm practice and during all stages of any disease response or ongoing management activities. Appropriate procedures are required to allow personnel, machinery and equipment to move safely between premises. Decontamination comprises planning, implementation and testing for efficacy (DAFF, 2008). This work aimed to contribute to improved management of *B. exitiosa* in oyster hatcheries and farms by providing guidelines for decontamination to ensure effective control of *B. exitiosa* on equipment and in water.

9.3 Objectives

We assessed the utility of sodium hypochlorite and Detsan Detergent-Sanitiser (a quaternary ammonium compound (QAC); permitted under APVMA PER82160 for use in oyster farms against OsHV-1. We tested these products against *B. exitiosa*. We used APVMA deactivation criteria (APVMA, 2014) and measured efficacy against purified *B. exitiosa* We assessed exposure time and concentration particularly following the APVMA validation of test methods (APVMA, 2014).

9.4 Methods

Ostrea angasi obtained from a farm at Grassy Point, Victoria, were held at the South Australian Aquatic Biosecurity Centre (SAABC) Roseworthy Campus, and maintained in 500 L tanks with continuous aeration for 21 months, fed every 2–3 days 15 L of mixed live algae culture (*Chaetoceros muelleri, Skeletonema costatum and Pavlova lutheri*) per tank. Testing animals from this group by heart smear showed 100% prevalence with light infection; *B. exitiosa* intensity was assessed using a scoring system from (Buss et al., 2018). Preference was made for oysters with slow closure response times and pale tissue coloration (translucent mantle and gill). Ten oysters were first pre-screened via heart smear following the protocol in Buss et al., (2018), with all tissue, excluding adductor muscle, maintained separately in 50 mL sterile, plastic tubes at 4°C. On the same day, tissue from seven oysters with highest heart smear intensity were further homogenised (TissueRuptor®, Qiagen) and used for purification. Purification followed a protocol from Diggles and Hine (2002), which was modified from Miahle et al. (1988). In the final step, purified parasites were re-suspended in autoclaved

(130°C for 30 min) filtered (0.2 µm) seawater (pH 7, salinity: 40 ppt), vortexed, distributed into 30 µL aliquots and maintained at 4°C, 12 h prior to use.

A ranging study based on the exposures in APVMA PER14029 was undertaken using sodium hypochlorite (NaOCl) (Chem-supply 8–12.5% available chlorine) at 1 ppm, 10 ppm, 100 ppm, 1000 ppm and 10,000 ppm. All dose measurements relate to available free chlorine. Free chlorine was measured using WaterWorks™ (Ionode, CAT: 480024 and 480022) test strips to ensure accuracy of exposures. 30 µL of purified *B. exitiosa* cells were exposed to equal volume of decontaminant and assessed for viability at 1 min, 5 min and 10 min exposure. A seawater control was included. After exposure to the decontaminant, the viability of *B. exitiosa* cells was assessed using trypan blue stain (0.4%, Sigma Aldrich, CAT: T8154), (filtered to 0.2 µm). Equal volume of trypan blue stain was added to the mixture, briefly vortexed and 10 µL was further loaded onto a haemocytometer and visualised under a compound light microscope at 400x. This process was replicated three times for each time and concentration treatment. Viable *B. exitiosa* cells were defined as cells that did not take up the stain whereas unviable cells did take up the blue stain (Figure 44) as per Diggles and Hine (2002). The 10,000 ppm sodium hypochlorite treatment interfered with the trypan blue stain and cell viability could not be analysed for that concentration.

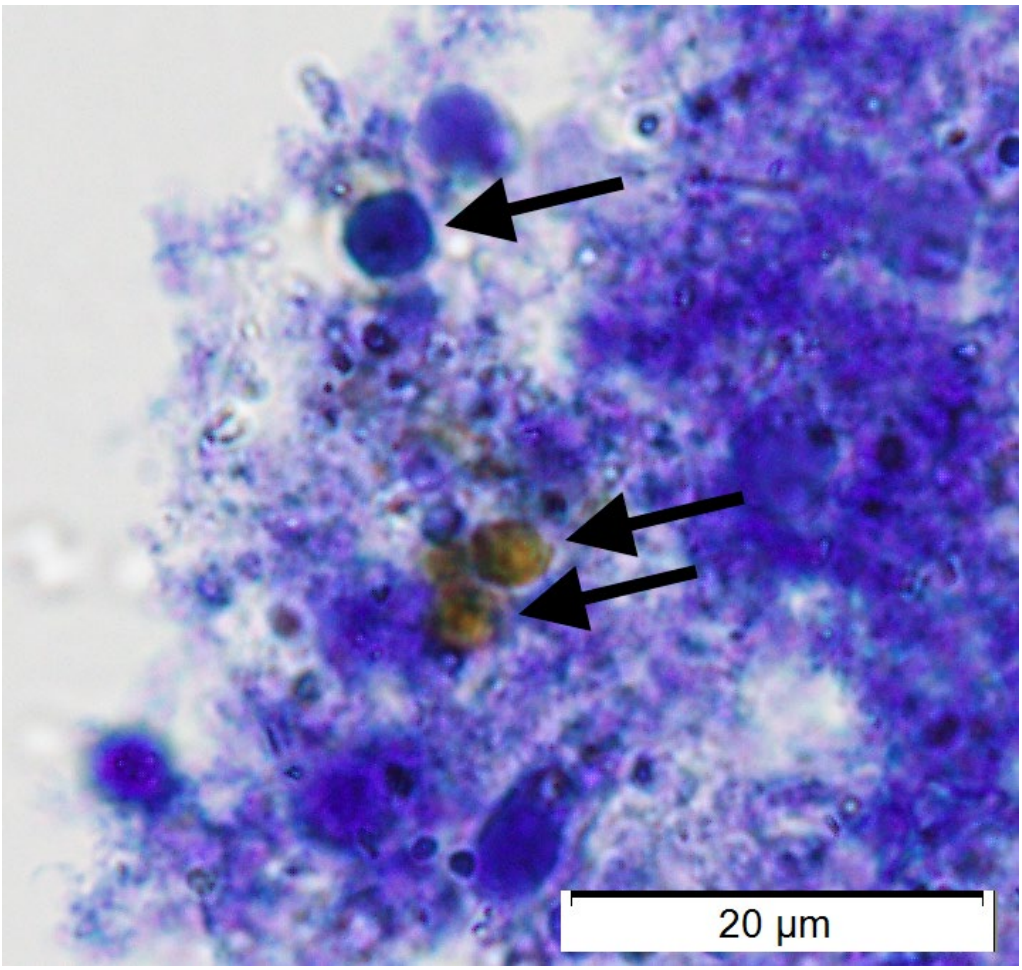

Figure 44. *Bonamia exitiosa* cell viability being assessed by trypan blue staining. Arrows point to viable cells (brown/golden) and a dead cell (blue)

A further study was undertaken using NaOCl at 10,000 ppm, 20,000 ppm and 40,000 ppm. We also assessed a quaternary ammonium compound (Detsan Detergent-Sanitiser, Chemetall) based on APVMA PER82160 at 10,000 ppm, 40,000 ppm and 80,000 ppm. Each

exposure was replicated three times. Each treatment was assessed at 1 min, 5 min and 10 min. To prevent hypochlorite interfering with the trypan blue stain, sodium thiosulphate was added to each sodium hypochlorite treatment at each sampling time period to neutralise the hypochlorite before staining and viability assessment. A seawater control was included. A sodium thiosulphate control was also included to assess if sodium thiosulphate had any effect on cell viability. The sodium thiosulphate control used seawater and the equivalent sodium thiosulphate that was added to the 40,000 ppm of NaOCl treatment. Sodium thiosulphate calculations were based from OIE (2009).

Cell viability = viable cells / mL / (viable cells / mL + dead cells / mL) * 100. Viability for each decontaminant treatment (QAC, NaOCl and sodium thiosulphate) were further expressed as a proportion of the seawater control viability. Efficacy per treatment was calculated as percentage reduction of mean cell viability using the following formula, modified from Stone et al. (2000): treatment efficacy = 100 – (100 * (mean cell viability of treatment / mean cell viability for the seawater control treatment).

Homogeneity of variance among means was assessed using Levene's test and the normality assessed with the Shapiro-Wilk test. In order to ensure normal distribution and homogeneity of variance, data were log transformed where appropriate. Two-factor analysis of variances (ANOVA) were used to assess the main effects (time and concentration) and interactions on cell viability for: NaOCl concentrations vs. seawater control in the ranging trial, QAC concentrations vs. sea water control and NaOCl concentrations vs. seawater and sodium thiosulphate controls. One-factor ANOVAs and Tukey HSD multiple comparison post-hoc tests were used to assess differences between treatments where an interaction was observed. All statistical analyses were done with IBM SPSS version 23 for Macintosh (IBM SPSS Inc., Chicago, IL).

9.5 Results

In the ranging study, *B. exitiosa* cell viability was significantly lower after exposure to 100 and 1000 ppm NaOCl treatments than cell viability after exposure to 10 ppm, 1 ppm NaOCl or the seawater control treatments ($p<0.001$, Figure 45). There was no significant difference in cell viability between sea water control concentrations at any time ($p>0.05$, Figure 45). There was no significant difference in cell viability between time treatments ($p=0.473$), but a significant interaction was observed between concentration and time ($p=0.013$); cell viability for sea water control at 1 min was significantly higher than the 1 ppm NaOCl treatment, whereas cell viability at 5 and 10 min for the sea water control, did not significantly differ to the 1 ppm NaOCl treatment (Figure 45).

In the ranging study, the 10,000 ppm sodium hypochlorite treatment could not be analysed for cell viability due to immediate bleaching of trypan blue stain. At this concentration, cells were still present, but all cells were bleached without staining and it was unclear if they were viable or dead. The 10,000 ppm treatment was omitted from further analyses in the ranging study. Maximum efficacy was only 70.72% for the highest concentration and longest exposure time treatment (1000 ppm for 10 min) (Table 30).

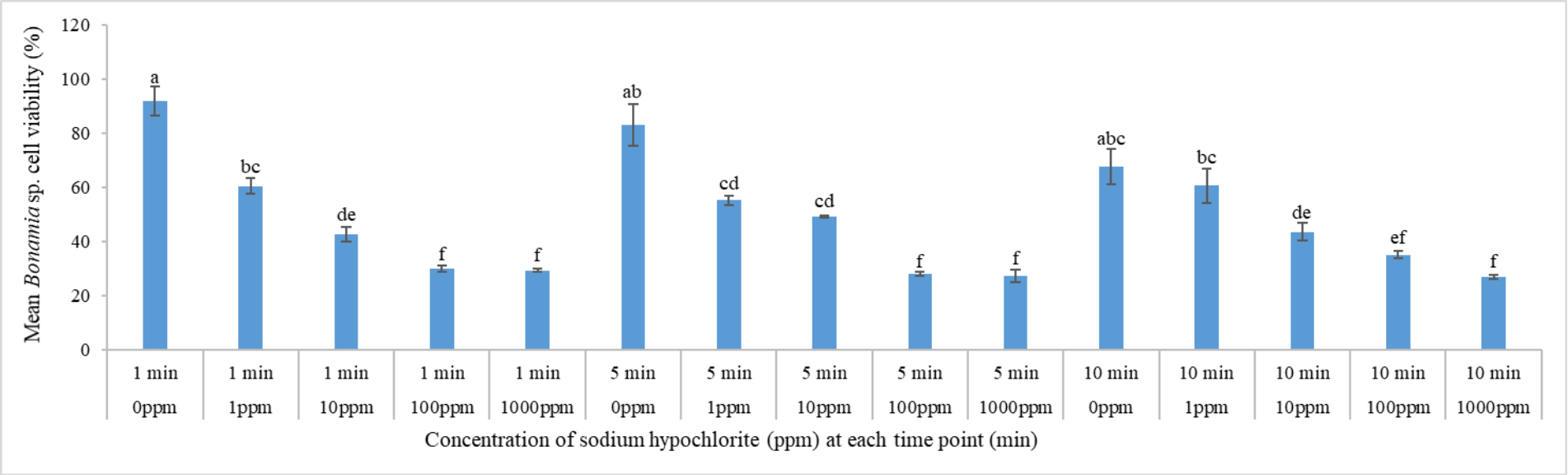

Figure 45. Mean *Bonamia exitiosa* cell viability (%) after exposure to different time and concentration treatments of sodium hypochlorite and a seawater control (0 ppm). Values were mean ± SE; n=3. Different superscripts denote significant differences ($p < 0.05$)

Table 30. Mean *Bonamia exitiosa* cell viability (%) and efficacy (%) per time and concentration treatment of sodium hypochlorite (NaOCl) or the seawater control (0 ppm) in the ranging study. Cell viability values were mean ± SE; n = 3

	Bonamia exitiosa cell viability (%)			Efficacy of treatment (%)		
NaOCl concentration	1 min	5 min	10 min	1 min	5 min	10 min
0 ppm	91.81±5.43	83.05±7.58	67.64±6.65	0.00	9.54	26.33
1 ppm	60.48±2.95	55.17±1.70	60.62±6.32	34.12	39.91	33.97
10 ppm	42.64±2.65	49.28±0.43	43.49±3.23	53.55	46.33	52.63
100 ppm	29.89±1.16	27.92±0.85	35.03±1.34	67.45	69.59	61.85
1000 ppm	29.26±0.65	27.26±2.17	26.88±0.84	68.13	70.31	70.72

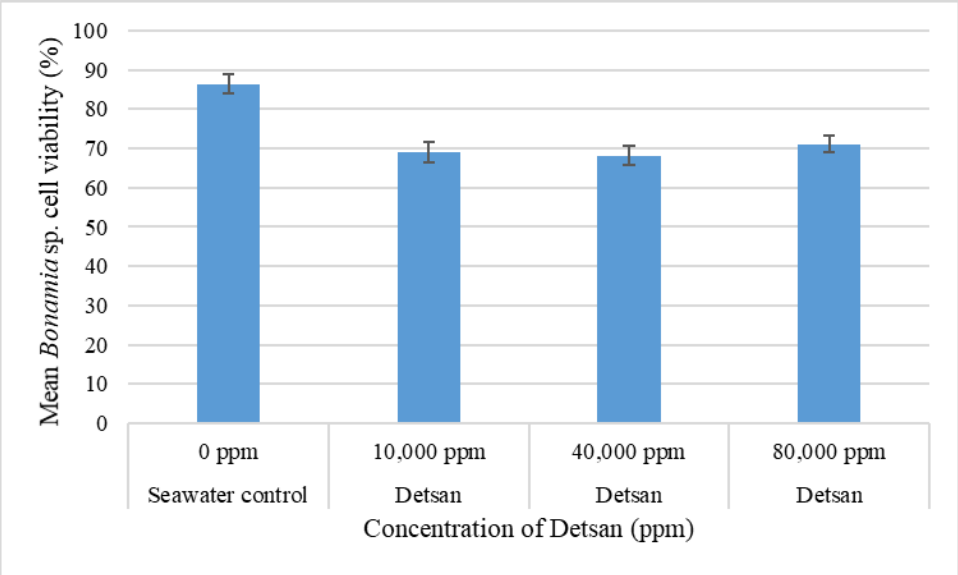

Figure 46. Mean *Bonamia exitiosa* cell viability (%) after exposure to different concentrations of QAC and seawater control (0 ppm QAC). Values were mean ± SE; n=3

Table 31. Mean *Bonamia exitiosa* cell viability (%) and efficacy (%) per time and concentration treatment of QAC or the seawater control (0 ppm) in the QAC assessment. Cell viability values were mean ± SE; n = 3

	Bonamia exitiosa cell viability (%)			Efficacy of treatment (%)		
QAC concentration	1 min	5 min	10 min	1 min	5 min	10 min
0 ppm	90.15±4.99	84.18±1.05	84.82±5.77	0.00	6.62	5.91
10,000 ppm	72.03±5.85	65.98±4.05	68.97±3.92	20.11	26.81	23.49
40,000 ppm	70.95±7.39	66.17±3.22	67.37±2.26	21.30	26.60	25.27
80,000 ppm	73.26±5.56	69.73±3.77	70.39±3.09	18.74	22.66	21.93

In the QAC assessment, *B. exitiosa* cell viability was significantly lower after exposure to any QAC concentration (10,000 ppm, 40,000 ppm or 80,000 ppm) than cell viability after exposure to the sea water control (p<0.001, Figure 46). After exposure, cell viability did not significantly differ between any QAC concentrations (p>0.05, Figure 46), with cell viability remaining high >65% (Table 31). There was no significant difference in cell viability between time treatments

(p=0.284) nor any interaction observed between concentration and time (p=1.000). Efficacy for all QAC treatments remained low (<26.81%, Table 31).

In the NaOCl assessment, *B. exitiosa* cell viability was significantly lower after exposure to any NaOCl concentration (10,000 ppm, 20,000 ppm and 40,000 ppm) than cell viability after exposure to the sea water or sodium thiosulphate controls (p<0.001, Figure 47). After exposure, the cell viability between all NaOCl concentrations (10,000 ppm, 20,000 ppm and 40,000 ppm) significantly differed between each other, with significant decreases in cell viability with increasing NaOCl concentration (p<0.001, Figure 47). After exposure, cell viability did not significantly differ between the sodium thiosulphate and seawater control (p>0.05, Figure 47). In the NaOCl assessment, there was no significant difference in cell viability between time treatments (p=0.138) nor any interaction observed between concentration and time (p=0.934). 100% efficacy was reached for the 40,000 ppm NaOCl treatment at 10 min (Table 32). The 5 min and 10 min exposure time treatments at 40,000 ppm NaOCl also had high efficacy >97% (Table 32).

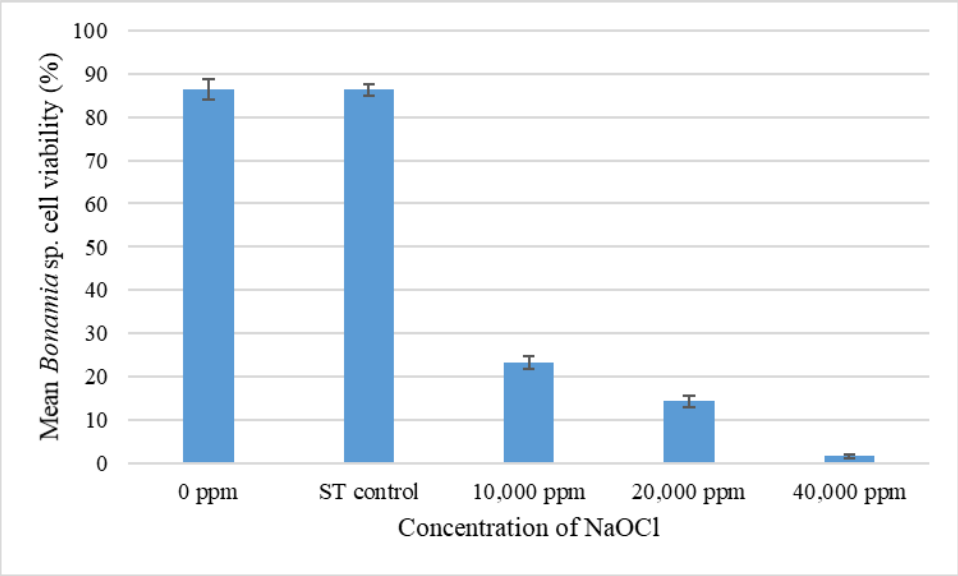

Figure 47. Mean Bonamia sp. cell viability (%) after exposure to different concentrations of sodium hypochlorite (NaOCl) or the sodium thiosulphate (ST) and seawater controls (0 ppm). Values were mean ± SE; n = 3

Table 32. Mean *Bonamia exitiosa* cell viability (%) and efficacy (%) per time and concentration treatment of sodium hypochlorite (NaOCl), the sodium thiosulphate (ST) or seawater (0 ppm) controls in the NaOCl assessment. Cell viability values were mean ± SE; n = 3

	Bonamia exitiosa cell viability (%)			Efficacy of treatment (%)		
NaOCl concentration	1 min	5 min	10 min	1 min	5 min	10 min
0 ppm	90.15±4.99	84.18±1.05	84.82±5.77	0.00	6.62	5.91
0 ppm (ST)	87.18±2.74	85.71±2.65	86.25±2.58	3.30	4.93	4.33
10,000 ppm	27.29±3.27	20.18±0.46	22.32±2.66	69.73	77.61	75.24
20,000 ppm	15.37±1.59	15.00±2.75	12.47±3.58	82.95	83.37	86.17
40,000 ppm	2.66±0.60	2.19±1.10	0.00±0.00	97.05	97.57	100.00

9.6 Discussion

The ranging study showed that hypochlorite had potential as a decontaminant, but exposure required further exploration and optimisation. Viable cells remained in all treatments and the highest efficacy was 70.72% for the 1000 ppm NaOCl at 10 min (Table 30).

Trypan blue staining was affected by sodium hypochlorite at concentrations over 1000 ppm. Sodium thiosulphate neutralisation prevented hypochlorite from interfering with staining and facilitated successful visualisation of live and dead *B. exitiosa* cells hypochlorite concentrations >10,000 ppm. The thiosulphate control showed that this treatment did not significantly affect cell viability (Figure 47, Table 32).

The NaOCl assessment found the optimal dose and time to effectively kill all viable *B. exitiosa* cells; 100% efficacy was reached for the 40,000 ppm NaOCl treatment at 10 min (Table 32), but, efficacies at 5 min and 10 min at 40,000 ppm NaOCl, were still high >97% (Table 32). PER14029 provides access for oyster growers to hypochlorite for decontamination, but only up to 1% available chlorine (10,000 ppm). The permit may require amendment for it to cover effective use of hypochlorite against *B. exitiosa.*

Quaternary ammonium compound is not a suitable decontaminant for *B. exitiosa.* It did significantly decrease cell viability with the highest mean efficacy at 26.81%, after 5 min exposure to 10,000 ppm QAC (Table 31), but this is not adequate efficacy to recommend it for routine use. Effective cleaning must always precede disinfection. Quaternary ammonium detergents are not, however, without utility. Thorough cleaning can remove more than 90% of pathogen loading on equipment (Fotheringham, 1995; Lewis, 1980) by removing organic material, biofilms and chemical residues (DAFF, 2008).

9.7 Conclusions

Quaternary ammonium compound is not suitable for decontaminating equipment exposed to *B. exitiosa but is sui*table for cleaning. Hypochlorite at 4% active chlorine for 10 minutes reliably decontaminates 100% of *B. exitiosa* cells and provides a useful basis for cleaning equipment.

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Implications

The information generated through this project has confirmed the ability of current PCR tests, in association with Sanger sequencing, to detect and identify *B. exitiosa* in *O. angasi*. The characterisation of *B. exitiosa* has been greatly expanded with the draft genome that has been assembled. The assembly of the Australian flat oyster (*O. angasi*) will provide a platform for further research that could aid the selection of characteristics favourable to farming.

The information generated through this project determined a number of risk factors for the development of clinical bonamiosis in Victorian Native Oysters. It is possible that some factors can be manipulated at a farm level and others, such as water temperature cannot. The project validated the CSIRO qPCR for *Bonamia* and identified the optimal C_T value.

The information developed in the SARDI component of the project provides a platform for *Bonamia* surveillance, understanding transmission to *O. angasi* and Pacific oysters, and indicates that some areas in South Australia may be more suitable for farming *O. angasi*. The information on decontaminants provides a basis for understanding the environmental robustness of *Bonamia* cells and is useful for decontaminating oyster farming equipment in a way that neutralises *Bonamia* cells.

Recommendations

Further development

This project has generated draft genomes for *B. exitiosa* and *O. angasi* and both assemblies require additional sequencing for completion of the genomes and gene identifications. Additional research would include further purification of *B. exitiosa* from infected flat oysters to obtain pure *B. exitiosa* DNA for sequencing. This would allow for more accurate genome assembly and genome size estimates based on flow cytometry or computational k-mer profiling. Moreover, sequencing the messenger RNA of infected oysters would provide valuable data for confirming the predicted *B. exitiosa* and *O. angasi* gene models, and can also be used as another measure of genome completeness. Moreover, a small amount of additional long-read sequence data (i.e. MinION NGS platform) could elevate both genomes to high-quality drafts. At this time there is not a species specific *Bonamia exitiosa* qPCR available however there is currently work underway in New Zealand to develop this test.

The infection models facilitate further work on *Bonamia*, including development of a breeding program for resistance. The SARDI laboratory and South Australian field trials indicate that this is likely to be necessary for the industry to expand substantially and profitably. The diagnostic performance information provides a basis for surveillance for *Bonamia*, and a surveillance program could be developed if industry began to suffer mortalities. The field trial indicates that environmental factors that are not described may influence farm infections. Further fine scale studies using better measurement of environmental factors and more thorough surveillance may provide a better understanding of why some areas are less conducive to *Bonamia* transmission. In combination with resistant stock, understanding

transmission could provide a basis for an expanding profitable industry to aid the edible oyster industries to diversify and avoid substantial commercial impacts such as those that occurred when OsHV-1 outbreaks negatively affected the profitability of the Pacific oyster industry.

APVMA PER14029 should be amended to include label directions for decontaminating equipment that is at risk of carrying *B. exitiosa*.

The strong association found in Victoria between origin of stock and risk of death due to clinical bonamiosis requires further investigation. Given the geographic isolation of these stock and the presumed differences in genetic heritage, some conferred differences in the response to the development of clinical bonamiosis is possible.

Although the field trial in Victoria was supported by farmers and had great application, the inability to closely monitor the health of oysters may have hampered the identification of clear risk factors for developing clinical bonamiosis. The repetition of this work with a greater level of control over health monitoring could help uncover purported risk factors.

Extension and Adoption

Draft genomes of *Bonamia exitiosa*, the Australian flat oyster, *Ostrea angasi* and the Epsilonproteobacterium, *Poseidonibacter* have been assembled for the first time. The tens of thousands of previously unknown sequenced genes are a valuable contribution to the literature. A peer-reviewed publication will be drafted detailing this achievement and allowing the dissemination of the data publicly through deposition of the sequences at the NCBI GenBank.

News Articles: Parasite infecting native oysters has 'come back to haunt us'. Cameron Best, ABC, updated 24 Jul 2016, 12:03pm

Animal Health Surveillance Quarterly – AHA Vol 20:4. Bonamiosis in farmed native oysters

News article "Bellarine Times" – 8/8/2016. "Queenscliff lab joins fight to save Native Oyster"

Presentation to Victorian Native Oyster farmers on the project outcomes 15/11/18, Queenscliff site.

Report on a "Visit to a Tasmanian Native Oyster farm – the Oyster Province, April 2018 (John Mercer and Tracey Bradley). Also provided in a presentation to Victorian Native Oyster farmers.

Peer – reviewed article currently in draft form detailing Victorian project results.

South Australian Oyster Growers Association (SAOGA) industry meeting. 3 August 2016. Cowell, South Australia. Buss J. J., Harris, J. O. & Deveney M. Presentation. *Bonamia* research on SA *Ostrea angasi.*

South Australian Oyster Growers Association (SAOGA) industry meeting. 4 August 2017. Coffin Bay, South Australia. Buss J. J., Harris, J.O. & Deveney M. Presentation. Update on *Bonamia* research on SA *Ostrea angasi.*

South Australian Oyster Growers Association (SAOGA) industry meeting. 22–24 August 2018. Smoky Bay, South Australia. Buss J. J., Harris, J. O. & Deveney M. Presentation. *Bonamia* research on SA *Ostrea angasi.*

Buss J. J., Harris, J. O. & Deveney M. Presentation and Conference Proceeding. Rapid transmission and pathogenesis of *Bonamia* infection in *Ostrea angasi*. Australian Marine Sciences Association, Canyons to Coast Conference. 1–5 July 2018. Adelaide Convention Centre, Australia.

Buss J. J., Wiltshire K., Prowse T., Harris J. O. & Deveney M. Presentation and Conference Proceeding. Survey of current prevalence and intensity of *Bonamia* sp. on South Australian *Ostrea angasi* hatchery and farm sites. Fourth Australian Scientific Conference on Aquatic Animal Health and Biosecurity. 10–14 July 2017, Cairns, Australia.

Buss J. J., Wiltshire K. H., Prowse T. A. A., Harris J. O. & Deveney M. R. (2018) Bonamia in *Ostrea angasi*: diagnostic performance, field prevalence and intensity. Journal of Fish Diseases 42(1), 63-74.

A workshop was planned to be delivered at a SAOGA meeting in March 2019. That meeting was cancelled due to extenuating circumstances, but the workshop was held at the next SAOGA general meeting in August 2019.

Draft Native Oyster Biosecurity Plan – produced as an activity within the project.

Project coverage

See above.

Appendices

1. Project staff

Name	Position	Organisation
Dr Mark Crane Dr Nick Moody Dr Peter Mohr Dr Matthew Neave Ms L Williams	Senior Principal Research Scientist Research Group Leader - AFDL Team Leader - Aquatic Diagnostic Capability Research Scientist Research Technician	CSIRO AAHL Fish Diseases Laboratory, Geelong, Victoria
Dr Tracey Bradley Dr Jaimie Hunnam Mr John Mercer Dr John Humphrey	Principal Veterinary Officer, Aquatic Animal Health Principal Veterinary Officer, Epidemiology Aquaculture Officer Pathologist	Agriculture Victoria, DJPR Victorian Fisheries Authority Panaquatic Health Solutions
Dr Marty Deveney Ms Kathryn Wiltshire Dr Jason Tanner Ms Jessica Buss Dr James Harris Dr Thomas Prowse	Marine Biosecurity Node Leader Senior Research Scientist-Biosecurity Subprogram Leader Marine Rehabilitation and PhD Student Associate Professor Senior Research Fellow	SARDI Aquatic Sciences Flinders University Flinders University The University of Adelaide

2. Intellectual property

Intellectual property generated by this project is in the form of draft genomes of *B. exitiosa*, *O. angasi* and *Poseidonibacter* spp. that have been assembled for the first time.

3. Conference proceedings

Bradley T., Moody N. & Crane M StJ. 2017. Presentation and Conference Proceeding. Description of an outbreak of Bonamia exitiosa in Victorian Native Oysters in Port Phillip Bay. Fourth Australian Scientific Conference on Aquatic Animal Health and Biosecurity. 10–14 July 2017, Cairns, Australia

Bradley T., Hunnam J. & Mercer J. 2018. Presentation and Conference Proceeding. *Bonamia exitiosa* in Australian Native Oysters. International Symposium on Aquatic Animal Health, September 2018, Charlottetown, Prince Edward Island, Canada.

Buss J. J., Harris, J.O. & Deveney M. 2018. Presentation. *Bonamia* research on SA *Ostrea angasi.* South Australian Oyster Growers Association (SAOGA) industry meeting. 22–24 August 2018. Smoky Bay, South Australia.

Buss J. J., Harris, J. O., & Deveney M. Presentation and Conference Proceeding. Rapid transmission and pathogenesis of *Bonamia* infection in *Ostrea angasi*. Australian Marine Sciences Association, Canyons to Coast Conference. 1–5 July 2018. Adelaide Convention Centre, Australia.

Buss J. J., Wiltshire K., Prowse T., Harris J. O. & Deveney M. 2017. Presentation and Conference Proceeding. Survey of current prevalence and intensity of *Bonamia* sp. on South Australian *Ostrea angasi* hatchery and farm sites. Fourth Australian Scientific Conference on Aquatic Animal Health and Biosecurity. 10–14 July 2017, Cairns, Australia.

Buss J. J. & Deveney M. 2016. Presentation. *Bonamia* in Native Oyster aquaculture in Australia. Workshop - Flat oyster (*Ostrea angasi*) aquaculture development in Australia, SARDI Aquatic Sciences. 22 June 2016.

Hunnam J. & Bradley T. 2017. Risk factors associated with the conversion from subclinical to clinical Bonamiosis in native oysters in Port Phillip Bay: Preliminary results from Year one of field and tank trials. Fourth Australian Scientific Conference on Aquatic Animal Health and Biosecurity. 10–14 July 2017, Cairns, Australia