Infection with *Bonamia exitiosa* in the Australian Native Oyster (*Ostrea angasi*).

By

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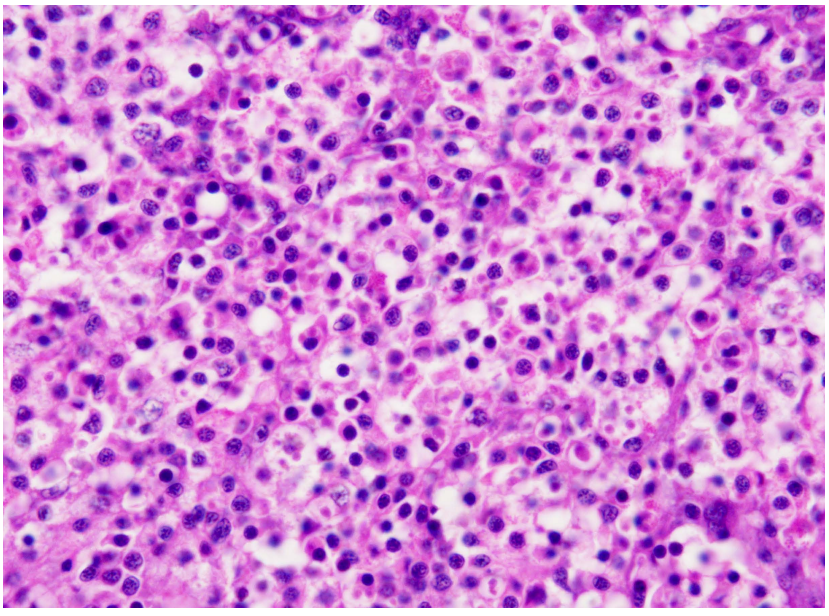
B.Sc. B.Tech. (Hons)



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Summary

Pathogenic Haplosporidia in *Bonamia* Pichot, Comps, Tigé, Grizel & Rabouin, 1980 cause epizootics in mollusc populations worldwide. A *Bonamia* sp. was described in *Ostrea angasi* Sowerby, 1871 from Port Phillip Bay, Victoria in 1991 and threatens the Australian *O. angasi* aquaculture industry. Most *Bonamia* spp. research originates in Europe, North America and New Zealand, leaving *Bonamia* in Australia poorly understood. A pilot survey of farmed *O. angasi* using a *Bonamia* quantitative polymerase chain reaction (qPCR) test detected a *Bonamia* sp. in South Australia in December 2015 and sequencing identified *Bonamia exitiosa* Hine, Cochennac & Berthe, 2001.

My study began with investigations of diagnostic performance and prevalence and intensity estimates. Surveys of three *O. angasi* farms in South Australia by histology, heart smear and qPCR were analysed using a latent class model to assess diagnostic sensitivity (DSe) and specificity (DSp) of single tests and test combinations. Histology had the highest DSe and DSp of any single test, but performance was improved by combining histology and qPCR and defining a case as positive if either test returned a positive result. Farmed *O. angasi* in South Australia have high *B. exitiosa* prevalence but low intensity of infection. Understanding diagnostic test performance informs design of better surveillance programs.

European *Bonamia ostreae* Pichot, Comps, Tigé, Grizel & Rabouin, 1980 infections in *Ostrea edulis* Linnaeus, 1758 build slowly, but infection dynamics of *B. exitiosa*-*O. angasi* were undescribed. An infection model was developed in which naïve recipient *O. angasi* were cohabited with *B. exitiosa* infected donor *O. angasi.* I monitored *B. exitiosa* prevalence and intensity in recipients over 40 days exposure. *Bonamia exitiosa* rapidly infects, and causes clinical disease and death in *O. angasi*. First infection is <10 days, and continuous exposure causes increasing *B. exitiosa* prevalence and intensity. Host death is not required for transmission. The infection model provides a basis for *B. exitiosa*-*O. angasi* studies including selecting *Bonamia* resistant oysters.

Seasonally variable infections are described for *Bonamia* spp.-oyster systems, but *B. exitiosa*-*O. angasi* infections were undescribed. Naïve *O. angasi* were placed on four *B. exitiosa* endemic South Australian farms and prevalence and intensity were monitored seasonally for one year. *Bonamia exitiosa* prevalence increased to >0.50 after one year. *Bonamia exitiosa* intensities were <4.0 after the first season but did not increase further. Sites with slower *B. exitiosa* prevalence increase may be preferable for *O. angasi* farming. *Bonamia exitiosa* must be considered in planning industry expansion and reef restoration.

Decontamination guidelines for *Bonamia* spp. were lacking. I assessed three disinfectants for dose-duration efficacy. An iodine and a chlorine based disinfectant provided 100% efficacy against *B. exitiosa*. Stability makes iodine based products preferred disinfectants. Effective decontamination guidelines for *B. exitiosa* can improve farm biosecurity and management decisions.

My research provides the first information on *B. exitiosa* in *O. angasi*. Information from my thesis creates a basis for future *B. exitiosa* research in Australia and provides the oyster industry with a foundation for informed decision making and clear direction for industry expansion.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Jessica Jamuna Buss

28 February 2020

Acknowledgements

Before all else, I happily acknowledge and thank my two supervisors, Assoc. Prof. Marty Deveney and Assoc. Prof. James Harris. Without them, I would not be writing this very sentence, let alone have embarked on this PhD journey. James' support began well before he became PhD supervisor: his endless enthusiasm, encouragement and approachable nature in my undergraduate years pursued me into honours and gave me the courage to apply for a PhD. Thank you for your attention to fine detail when preparing manuscripts, your comical humour which uplifted the lab in times when we needed it most and career advice beyond 'the void'. Marty has been at the forefront in introducing me to disease epidemiology; with my background in abalone nutrition and initial lack of molecular knowledge, it is remarkable he did not transform into a *Benedenia sciaenae* van Beneden, 1856 in my first year. Marty provided many travel opportunities to other scientific institutes, laboratories, and industry meetings, guiding me in all skills I needed to complete this PhD. His patience, generosity with time and inclusiveness into an industry setting has truly prepared me for future transfer into working life. Thank you for making my first draft manuscripts run red, your unwavering confidence in me and for understanding my numerous German colloquialisms; in my mind, pincers remain a perfectly suitable term for forceps, stemming from the German word, Pinzetten.

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In the last three years, I sampled more than 5,000 oysters. Despite becoming the official 'oyster shucker' at parties, the amount of preparation this takes is inconceivable. I feel fortunate to have had the network support of my family and friends, who gave their time to attend slide polishing, tube and histology cassette labelling 'parties' as well as their help on sampling days. Special thanks go to Imogen Guthrie, Sarah Biermann, Lauren Houthyusen, Kate McNaughton, Anne Andrews, Helen Charlton and Jess Simmons. Jess, thank you for your daily encouragement, support and friendship. Who knew electrical engineering would lead you down the path of learning oyster anatomy. You were my rock during the PhD and I am forever grateful.

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in the wonders and curiosities of the ocean. Josh, I remember following you in awe, trying to absorb your knowledge of fish. I still reminisce you helping me set up my first aquarium at an early age; between Papa, you and I, we definitely drove Mama up the wall with our aquariums, I fondly recall having up to eight aquariums in the house at one time. At least Mama can now relax, knowing the high electricity bills were all for a good cause.

They say it takes a family to finish a PhD and I feel this saying resonates true in my case. To all those not printed on paper, you know who you are. I thank you from the microscopic world of parasitism concealed within the dark voids of the ocean.

Chapter 1 : General Introduction

Figure 1: Moonset over the oyster leases, moments before sunrise, Streaky Bay, South Australia.

1.1 Introduction

1.1.1 Edible oysters in South Australia

Oyster industries are economically, socially and environmentally important in Australia. The earliest evidence of oyster harvesting in Australia is in coastal shellfish middens that are at least 10,000 years old (Godfrey, 1989). Midden content is site dependent and reflects the local marine environment (Stockton, 1977). Middens generally comprise shells of mixed bivalve and gastropod species and provide insights into historical diet, cultural practices and significance of molluscs to coastal Aboriginal people (Stockton, 1977). For Aboriginal people, shellfish were important beyond dietary needs, and were used to manufacture fish hooks, cutting tools (Stockton, 1977), jewellery (Colley, 2005), as a trade resource and for ceremonies (Clune & Harrison, 2009).

In the Australian colonial period, oysters were a food source and their harvest provided careers in fishing, boat building (Shefi, 2006), fish mongering and at oyster bars (Gillies et al., 2018). Increasing European population in Australia motivated extensive dredging of oyster reefs for food, and middens were mined for lime (Nell, 2001). No extensive pre-colonisation reefs remain in southern Australia (Alleway & Connell, 2015; Beck et al., 2011) and reef restoration programs aim to re-establish them in the 21st century (Gillies, Crawford, & Hancock, 2017; Gillies et al., 2018; Ford & Hamer, 2016). The Australian Native Oyster (*Ostrea angasi* Sowerby, 1871) is of substantial interest for reef restoration (Gillies et al., 2017).

1.1.2 Early Australian oyster aquaculture and development of the South Australian oyster industry

Australian oyster aquaculture is dominated by *Crassostrea gigas* Thunberg, 1793 (see Gillies et al., 2018; Jenkins et al., 2013; Maguire & Nell, 2007), except in Victoria where most farms cultivate *O. angasi* (see Bradley, 2019). *Saccostrea glomerata* Gould, 1850 is farmed predominately in New South Wales and to a lesser extent in Queensland and Western Australia (Gillies et al., 2018; Schrobback, Pascoe, & Coglan 2014). Production of *O. angasi* is substantially lower than that of *C. gigas* or *S. glomerata* (see Gillies et al., 2018; O'Connor & Dove, 2009).

Early European records of dredge and/or hand harvest of *O. angasi* or *S. glomerata* include 1788 in Sydney, 1804 in Hobart, 1824 in Brisbane, 1835 in Melbourne and 1836 in Adelaide (Gillies et al., 2018). Stocks were depleted by overfishing by the late 1800s to early 1900s, shifting the focus to aquaculture. The first attempts at oyster aquaculture in Australia began in 1870 with *S. glomerata* in New South Wales and southern Queensland (Nell, 2001). Culture methods were primitive; oysters were dredged and translocated to intertidal structures on mudflats (Nell, 2001). Problems with siltation and oyster shell infestation with spionid mud worms led to development of

stick and tray culture in the early 1900s (Maguire & Nell, 2007; Nell, 2001). In Tasmania, Victoria, South Australia and Western Australia, *O. angasi* aquaculture was attempted from the 1880s, but was abandoned by the 1890s (Nell, 2001; Warnock & Cook, 2015) due to low recruitment (Olsen, 1994).

After the formation of the Australian Division of Fisheries (now part of the Commonwealth Scientific and Industrial Research Organisation) in 1939, development of the Australian oyster aquaculture industry was prioritised (Thomson, 1952). The success of *C. gigas* importation to North America motivated consideration of importation of *C. gigas* for aquaculture trials in Australia. Between 1940 and 1970, *C. gigas* were periodically imported from Japan to Australia, and were distributed to Tasmania, Western Australia, Victoria and South Australia (Figure 1.1) (Coleman, 1986; Nell, 2001; Thomson, 1952; Thomson, 1959). Victoria had high initial survival, but Japanese stock were ultimately unsuccessful there and in South Australia and Western Australia (Coleman, 1986; Nell, 2001; Thomson, 1952; Thomson, 1959). Successful *C. gigas* farming required substantial recruitment, fast growth and high survival, which occurred only in Tasmania (Coleman, 1986; Nell, 2001; Thomson, 1952; Thomson, 1959). By the 1960s *C. gigas* aquaculture was established in Tasmania, using the stick and tray system developed for the New South Wales *S. glomerata* industry (Nell, 2001). Successful *C. gigas* aquaculture in Tasmania facilitated further *C. gigas* aquaculture trials in South Australia (Figure 1.1), which demonstrated high growth, but poor spat settlement, which at the time was presumed to be because of high local salinity (Medcof & Wolf, 1975; Olsen, 1994). By the early 1970s, the nascent South Australian industry was developing based on obtaining *C. gigas* spat from Tasmania for grow-out. Importation of *C. gigas* from Japan ceased in 1970 (Olsen, 1994) (Figure 1.1). The first Tasmanian *C. gigas* hatchery opened in 1981 and facilitated rapid industry expansion, including development of intertidal culture systems (English, Maguire, & Ward, 2000; Nell, 2001; Olsen, 1994). In the 1980s South Australian oyster industry development occurred in parallel with growing Tasmanian hatchery production (Nell, 2001). The first *C. gigas* hatchery in South Australia was built in the 1990s but industry-wide reliance on Tasmanian stock continued (Nell, 2001). South Australian farmers predominantly use an adjustable long-line system developed in South Australia (Wear, Theil, Bryars, Tanner, & de Jong, 2004). In Australia, edible oysters are the third most valuable aquaculture sector, worth $AUD 112 million with most production in South Australia ($AUD 40 million), Tasmania ($AUD 45 million) and New South Wales ($AUD 26 million) (Mobsby, 2018), producing approximately 11,000 tonnes (Mobsby & Koduah, 2017).

Crassostrea gigas has experienced epizootics in southern Australia caused by ostreid herpes virus-1 microvariant (OsHV-1 microvariant) (Castinel et al., 2015), but *O. angasi* is not susceptible

to disease caused by OsHV-1 (see Kirkland, Hick, & Gu, 2015). Farming *O. angasi* can therefore provide an alternative approach to managing OsHV-1 disease, but limits to widespread and successful *O. angasi* cultivation, including disease management, must be addressed first.

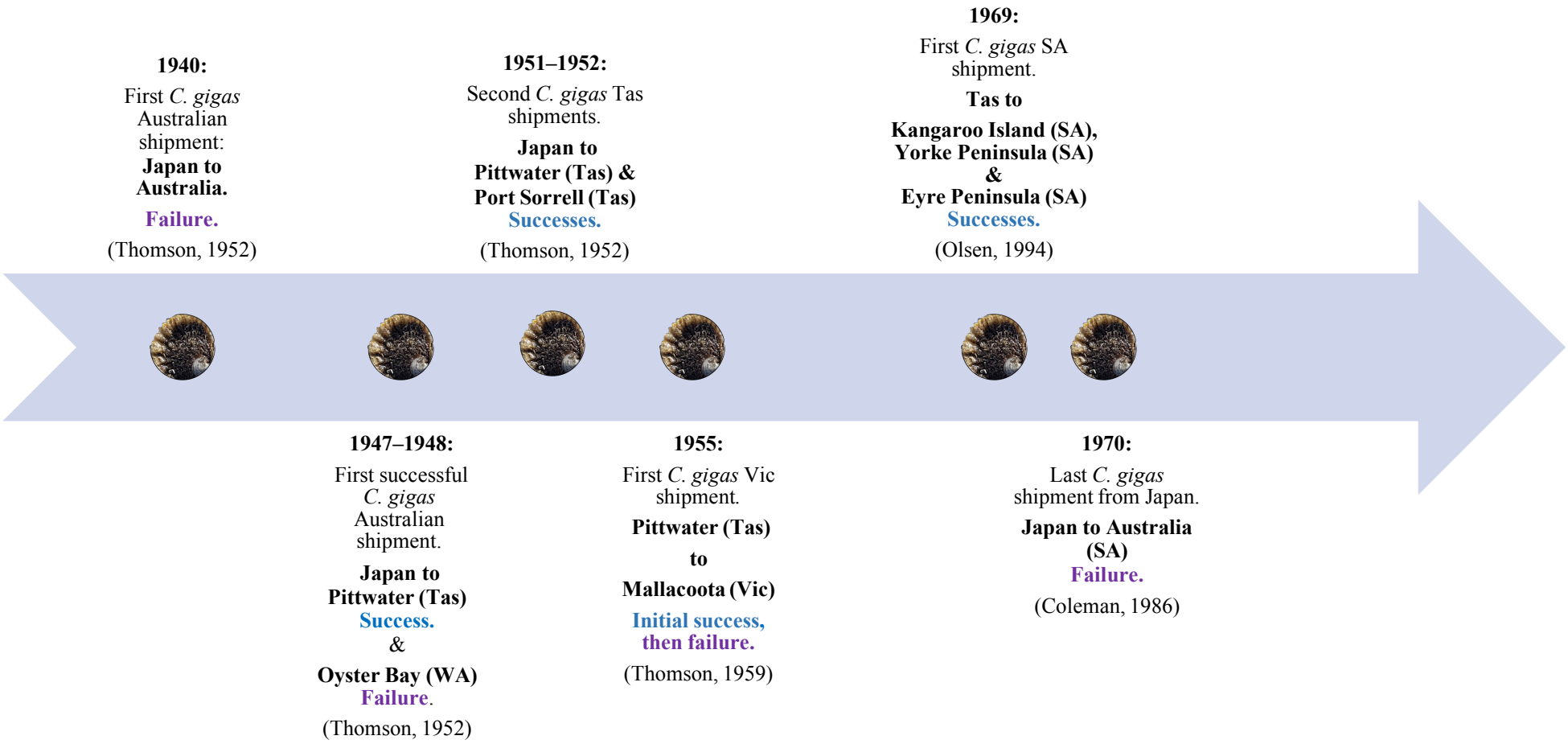

Figure 1.1: Timeline of *Crassostrea gigas* shipments into Tasmania (Tas), Victoria (Vic) and South Australia (SA). Success = oysters survived shipment and stocking in new area. Failure = oysters did not survive shipment and stocking in new area.

1.1.3 History of Australian Ostrea angasi *aquaculture*

In the 1970s, there was renewed interest in *O. angasi* as an aquaculture species in Australia; larval collection and rearing were attempted in Tasmania (Dix, 1976). *Ostrea angasi* are sequential protandric hermaphrodites that brood larvae for approximately 15–18 days followed by a planktonic larval phase of 10–15 days (O'Sullivan, 1980). Individual oysters can change sex within a season, which complicates selection of breeding pairs. The lower fecundity of brooding oysters compared to broadcast spawning oysters also makes hatchery production more difficult (O'Connor, 2015). In the 1980s, the *O. angasi* breeding season in South Australia was described (O'Sullivan, 1980) and further culture attempts were made in Tasmania (Dix, 1980) and Victoria (Hickman, 1984). The first *O. angasi* hatchery and nursery was built in Victoria in the mid-80s had successful spawning, high larval survival and settlement and good spat growth (Hickman, O' Meley, & Morris, 1987; Hickman & O'Meley, 1988). Research into optimised culture methods (Johnstone, 1986), storage and shelf life (Mantzaris, Hickman, & Grossel, 1991) continued until 1991, when a disease epidemic in Port Phillip Bay caused by a *Bonamia* sp. halted development (Handlinger et al., 1999; Hine & Jones, 1994). In Western Australia, an *O. angasi* hatchery was built and grow-out trials began in the 1990s (Warnock & Cook, 2015), but *Bonamia* sp. outbreaks in 1993 also caused the abandonment of *O. angasi* cultivation there (Handlinger et al., 1999; Hine & Jones, 1994). In response to the failure of *O. angasi* cultivation, *S. glomerata* farming was developed in Western Australia (Warnock & Cook, 2015). Other challenges in *O. angasi* industry development include problems with controlled breeding for domestication of brooding oysters, poor post-harvest shelf life and weak sales in a market dominated by cupped oysters (Heasman & Lyall, 2000). In 1998, renewed *O. angasi* culture trials began in New South Wales (Heasman & Lyall, 2000) and hatchery production commenced at the Port Stephens Fisheries Institute with no reports of serious disease (O'Connor & Dove, 2009). Interest in farming *O. angasi* is ongoing; small scale *O. angasi* cultivation occurs in Tasmania, Victoria, New South Wales and South Australia (Li & Miller-Ezzy, 2017). Limitations to expanding *O. angasi* aquaculture include disease, spat supply, suboptimal culture systems and husbandry practices, short shelf life and limited markets (Li & Miller-Ezzy, 2017). Disease in Australian *O. angasi* aquaculture has predominantly been associated with *Bonamia*. In Western Australia, a herpes-like virus was detected by electron microscopy (Corbeil et al., 2009; Hine & Thorne, 1997) but its role in disease is unclear. No other pathogens have been described from *O. angasi* in Australia. Development of a breeding program to improve survival, growth and aesthetic traits would accelerate industry development (Li & Miller-Ezzy, 2017).

1.1.4 Bonamia

Bonamia spp. are haplosporidian protozoan parasites responsible for mortalities in oyster populations globally (Engelsma, Culloty, Lynch, Arzul, & Carnegie, 2014). *Bonamia exitiosa* Hine, Cochennac & Berthe, 2001 and *Bonamia ostreae* Pichot, Comps, Tigé, Grizel & Rabouin, 1980 are listed as notifiable by the World Organisation for Animal Health (OIE) (OIE, 2019a, b) due to their risk of international spread, absence from some countries and ability to cause severe disease (OIE, 2019c).

Bonamia ostreae, the type species of *Bonamia* was described in 1979 in Brittany, France, after a mass mortality of farmed *Ostrea edulis* Linnaeus, 1758 (see Arzul et al., 2006). After this epizootic, the French *O. edulis* industry collapsed (Hudson & Hill, 1991) with massive economic losses (Arzul et al., 2006). *Bonamia ostreae* is now distributed throughout the Atlantic coast of Europe (Arzul & Carnegie, 2015), the United States of America (Friedman & Perkins, 1994) and has heavily impacted farm and wild *O. edulis* populations (Arzul & Carnegie, 2015). From 1986–1992, the Foveaux Strait *Ostrea chilensis* Küster, 1844 fishery in New Zealand suffered mass mortalities caused by *B. exitiosa* infection (Cranfield, Dunn, Doonan, & Michael, 2005), in which the population declined to <10% of pre-disease biomass (Cranfield et al., 2005; Doonan, Cranfield, & Michael, 1994). The *O. chilensis* fishery was closed in 1993 to allow oyster recruitment to recover and reopened in 1996. *Ostrea chilensis* in Foveaux Strait suffered a second significant *B. exitiosa* epizootic from 2000–2005, in which the population declined to as low as were observed in the early 1990s (Michael, Forman, Hulston, Fu, & Maas, 2015). Mortality from *B. exitiosa* remains a driver of the productivity of the Foveaux fishery; increases in *O. chilensis* population density increases transmission and drive *B. exitiosa* mortality (see Fu, Dunn, Michael, & Hills, 2016). The *O. chilensis* population has never recovered to historical highs and after each successive epizootic the population stabilises at a lower biomass than before the preceding epizootic (Fu, et al., 2016). *Bonamia exitiosa* has also been detected in oysters from North America, South America, the Mediterranean and Australia (Hill et al., 2014). The negative impacts of *Bonamia* spp. on oyster industries worldwide are, therefore, widespread and severe.

Since the description of *B. ostreae*, three other *Bonamia* species have been described: *Bonamia roughleyi* Farley, Wolf & Elston, 1988, *B. exitiosa* and *Bonamia perspora* Carnegie, Burreson, Hine, Stokes, Audemard, Bishop & Peterson, 2006, but phylogenetic analysis does not support *B. roughleyi* as valid (Hill et al., 2014). Evidence that *S. glomerata* microcells sometimes associated with winter mortality are *Bonamia* sp. remains lacking (Spiers et al., 2014). A Hawaiian *Bonamia* isolate from *Dendostrea sandvichensis* Sowerby, 1871 is a new species that is basal to the

Bonamia clade and numerous lineages make up a clade that comprises isolates described as *B. exitiosa* (see Hill et al., 2014). There is undoubtedly greater diversity in *Bonamia* than described.

Bonamia spp. infect oyster hemocytes, which are the primary cellular defence for molluscs against pathogens and function primarily by phagocytosis, respiratory burst and apoptosis (Hughs, Foster, Grewal, & Sokolova, 2010). Transmission of *Bonamia* spp. can occur directly (Arzul & Carnegie, 2015; Carnegie et al., 2006), but the possible role of an intermediate host in the lifecycle of *Bonamia* spp. cannot be dismissed (Arzul & Carnegie, 2015).

After a pathogen is phagocytosed by a hemocyte, a combination of hydrolytic enzymes and reactive oxygen species (ROS) mediate destruction of the foreign organism (Hughs et al., 2010). *Bonamia* spp. and oyster hemocytes play active roles in internalisation (Chagot et al., 1992; Morga, Arzul, Chollet, & Renault, 2009). Infection is influenced by the ability of the parasite to infect a particular host and protect itself from destruction; after phagocytosis by *Crassostrea virginica* Gmelin, 1791 hemocytes, *Perkinsus marinus* (Mackin, Owen, & Collier) Levine, 1978 produces antioxidants including superoxide dismutase and peroxidase to prevent destruction of the parasite by host ROS processes (Soudant, Chu, & Volety, 2013). Parasite-hemocyte interaction is also a strong mediator of the disease caused by *Bonamia* spp. *Bonamia* spp. predominantly causes disease in *Ostrea* spp. (see Arzul & Carnegie, 2015; Lane, Webb, & Duncan, 2016). *Crassostrea* spp. and *Saccostrea* spp. are also hosts (Hill et al., 2014; Lynch et al., 2010), but *Bonamia* spp.-associated pathogenesis is less well established for these hosts (Lynch et al., 2010; Spiers et al., 2014). In particular, *C. gigas* appears to become infected by *B. ostreae* but the parasite does not cause disease (Lynch et al., 2010). Gervais, Renault, and Arzul (2018) found that *B. ostreae* cells could activate expression of the *O. edulis* gene inhibitor of apoptosis (IAP), allowing *B. ostreae* cells to divide and survive in *O. edulis* hemocytes. Gervais, Chollet, Renault and Arzul (2016) and Comesaña et al. (2012), however, found that the hemocytic response of *C. gigas* to *B. ostreae* decreases susceptibility. *Crassostrea gigas* and *O. edulis* induce apoptosis when infected with *B. ostreae*, however the apoptotic response to live *B. ostreae* cells is higher in *O. edulis* than *C. gigas* (see Gervais et al., 2016) and *C. gigas* have stable, low total hemocyte counts and greater respiratory burst capacity than *O. edulis* (see Comesaña et al., 2012). These differences allow *C. gigas* to control *Bonamia* spp. infection while *O. edulis* become diseased due to hemocyte destruction and die. Host cellular mechanisms are, therefore, a strong determinant of infection dynamics and pathogenesis of *Bonamia* spp.

Prior exposure of host molluscs to a pathogen can also decrease susceptibility to infection (Green & Speck, 2018); an interferon-like response is demonstrated in *C. gigas* exposed to OsHV-1 or the viral mimic polyI:C and the offspring of primed individuals also display increased immune

capacity (Green & Speck, 2018). The disease response mounted by hosts in areas that have been infected by *Bonamia* spp. for a long time differs from areas with no previous exposure; *Ostrea edulis* from *B. ostreae* free areas have higher susceptibility to *B. ostreae* infection than *O. edulis* from *B. ostreae* endemic areas (Culloty, Cronin, & Mulcahy, 2004). When *O. angasi* were introduced to France in an attempt to restore the depleted *O. edulis* industry, *O. angasi* contracted *B. ostreae* infection and this species was not considered a viable substitute (Bougrier, Bachere, & Grizel, 1986). Understanding infections and history of exposure to hosts in an area is therefore important for contextualising host responses to infection.

Research on *Bonamia* spp. has been focused on Europe, North America and New Zealand (Arzul & Carnegie, 2015; Engelsma et al., 2014). *Bonamia* sp. has been described in Victorian, Tasmanian, Western Australian (Corbeil, Handlinger, & Crane, 2009; Hine & Jones, 1994) and South Australian *O. angasi* populations (Buss, Wiltshire, Prowse, Harris, & Deveney, 2019) and isolates in southern Australia have been characterised as *B. exitiosa* (Bradley, 2019).

There was no information on *B. exitiosa* infection in *O. angasi*, however, which puts the Australian oyster industry in a challenging position. Reef restoration projects with *O. angasi* have commenced in South Australia (Gillies et al., 2017) and *O. angasi* has had renewed interest for aquaculture industry expansion (Kirkland et al., 2015). More information on infection dynamics of *B. exitiosa* in *O. angasi* was therefore needed. Information on *B. exitiosa* would inform planning surveillance and industry decisions about expansion of *O. angasi* culture and placement of restored or restocked reefs. *Bonamia exitiosa* infection in South Australian *O. angasi* is therefore the focus of this thesis.

1.2 Study objectives

The primary objective of this thesis was to improve knowledge of the *B. exitiosa* infections in *O. angasi* in South Australia. More specifically, six research aims were identified:

1. To confirm *B. exitiosa* infections in farmed South Australian *O. angasi* populations and assess prevalence and intensity of infection.
2. To assess performance of diagnostic tests to aid surveillance and monitoring program design and interpretation of test results for the Australian *O. angasi* industry.
3. To create a reliable laboratory infection model for *B. exitiosa*-*O. angasi* studies.
4. To assess time to first *B. exitiosa* infection in *O. angasi* and understand prevalence and intensity over time.

5. To identify seasonal and/or regional changes in *B. exitiosa* infections in farmed *O. angasi* populations.
6. To identify reliable decontaminants for *B. exitiosa*.

1.3 Thesis outline

This thesis encompasses six chapters: introduction, four data chapters and a general discussion. Data Chapters 2–5 have been published or are currently under review in Journal of Fish Diseases or Aquaculture. For continuity, all chapters are presented in the Journal of Fish Diseases style.

Chapter 2: *Bonamia* in *Ostrea angasi*: diagnostic performance, field prevalence and intensity. This chapter was published in the Journal of Fish Diseases (Buss et al., 2019) and addresses aims 1 and 2

Chapter 3: Rapid transmission of *Bonamia exitiosa* by cohabitation causes mortality in *Ostrea angasi*. This chapter addresses research aims 3 and 4 and is accepted for publication in the Journal of Fish Diseases (Buss, Harris, Tanner, Wiltshire, & Deveney, 2020a) and addresses aims 3 and 4.

Chapter 4: Infection dynamics of *Bonamia exitiosa* on intertidal *Ostrea angasi* farms. This chapter addresses research aim 5 and is accepted for publication in the Journal of Fish Diseases. (Buss, Wiltshire, Harris, Tanner, & Deveney, 2020b).

Chapter 5: Decontamination of *Bonamia exitiosa*. This chapter addresses research aim 6 and has been submitted to Aquaculture for publication and is currently under review.

1.4 Publications

1.4.1 Co-authorship of chapters

Chapters 2, 3, 4 and 5 are presented in a manuscript style suitable for publication in a scientific journal. All data chapters have been accepted or submitted for publication in the Journal of Fish Diseases or Aquaculture. There is therefore some repetition between chapters, particularly in the introductions and methods. I have written all chapters in this thesis and conducted all experimental work, diagnostic sampling, sample analyses and a majority of data analyses, but each chapter was co-authored due to contributions by my supervisors and other professionals. Assoc. Prof. James Harris and Assoc. Prof. Marty Deveney make up my supervisory team and are co-authors in each chapter due to their major contributions in direction, experimental design and manuscript preparation. Ms Kathryn Wiltshire and Dr Thomas Prowse are co-authors in Chapter 2, due to their contribution in data analysis. Ms Kathryn Wilshire is also a co-author in Chapters 3–5,

due to contributions in data analysis and assistance with manuscript preparation. Dr Jason Tanner is a co-author in Chapters 3 and 4, due to contributions with data analysis.

1.4.2 Thesis publications

Chapter 2: Buss, J. J., Wiltshire, K. H., Prowse, T. A. A., Harris, J. O., & Deveney, M. R. (2019). *Bonamia* in *Ostrea angasi*: Diagnostic performance, field prevalence and intensity. *Journal of Fish Diseases*, *42*, 63–72. doi: 10.1111/jfd.12906

Chapter 2: Buss, J. J. (2019) Back cover journal image: *Bonamia* in *Ostrea angasi*: Diagnostic performance, field prevalence and intensity. *Journal of Fish Diseases*, *42*. doi:10.1111/jfd.12948

Chapter 3: Buss, J. J., Harris, J. O., Tanner, J. E., Wiltshire, K. H., & Deveney, M. R. (2020a). Rapid transmission of *Bonamia exitiosa* by cohabitation causes mortality in *Ostrea angasi*. *Journal of Fish Diseases*, *43*, 227–237, doi: 10.1111/jfd.13116

Chapter 4: Buss, J. J., Wiltshire, K. H., Harris, J. O., Tanner, J. E., & Deveney, M. R. (2020b). Infection dynamics of *Bonamia exitiosa* on intertidal *Ostrea angasi* farms. *Journal of Fish Diseases*. *43*, 359–369, doi: 10.1111/jfd.13134

Chapter 5: Buss, J. J., Wiltshire, K. H., Harris, J. O., & Deveney, M. R. (under review, submitted 27 October 2019). Decontamination of *Bonamia exitiosa*. *Aquaculture.*

Chapter 2 : *Bonamia* in *Ostrea angasi*: diagnostic performance, field prevalence and intensity.

Figure 2: Oyster leases, Coffin Bay, South Australia.

Buss, J. J., Wiltshire, K. H., Prowse, T. A. A., Harris, J. O., & Deveney, M. R. (2019). *Bonamia* in *Ostrea angasi*: Diagnostic performance, field prevalence and intensity. *Journal of Fish Diseases*, *42*, 63–72. doi: 10.1111/jfd.12906

2.1 Abstract

Bonamia spp. parasites threaten flat oyster (*Ostrea* spp.) farming worldwide. Following a pilot survey which found low *Bonamia exitiosa* Hine, Cochennac, & Berthe, 2001 intensity in farmed *Ostrea angasi* Sowerby, 1871, we tested oysters (n = 100–150) from three farms for *B. exitiosa* using heart smear, histology and quantitative polymerase chain reaction (qPCR). Understanding test performance is important for surveillance design and interpreting diagnostic results. We used a Bayesian Latent Class Model to assess diagnostic sensitivity (DSe) and specificity (DSp) of these tests individually or in combination, and to estimate prevalence. Histology was the best individual test (DSe 0.76, DSp 0.93) compared to 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 tests when compared to other studies. Understanding diagnostic test performance in different populations can support development of improved *B. exitiosa* surveillance programs.

2.2 Introduction

Oysters have been an important food source in Australia for over two centuries, beginning with wild harvest and continuing with aquaculture. Pacific oyster (*Crassostrea gigas* Thunberg, 1793) farming has become the main edible oyster industry (Nell, 2001). The Pacific oyster industry in Australia, however, has suffered substantial production losses since 2010 caused by the emergence of the ostreid herpes virus-1 (OsHV-1) microvariant (see Castinel et al., 2015). Species diversification, including farming the Native Oyster (*Ostrea angasi* Sowerby, 1871), which is not susceptible to OsHV-1 microvariant (Kirkland, Hick, & Gu, 2015), is a strategy to improve industry resilience. Wild *O. angasi* populations and their role as ecosystem engineers have generated further interest in reef restoration projects (Gillies, Crawford, & Hancock, 2017). Attempts to cultivate *O. angasi* in the 1990s in Australia stalled due to disease, limited breeding technology and lack of suitable farming methods (Nell, 2001; O'Connor & Dove, 2009). Understanding disease risks is therefore important for assessing the viability of expansion of *O. angasi* aquaculture and restoration of wild populations.

Infection by haplosporidian protozoan parasites, *Bonamia* spp., have caused negative impacts on ostreine oysters worldwide (Baud, Gérard, & Naciri-Graven, 1997; Hine & Jones, 1994; Hudson & Hill, 1991), including *Ostrea chilensis* Küster, 1844*, Ostrea edulis* Linnaeus, 1758, *O. angasi*, *Ostrea stentina* Payraudeau, 1826 and *Ostrea puelchana* d'Orbigny, 1842 (see Engelsma, Culloty, Lynch, Arzul, & Carnegie, 2014; Hine & Jones, 1994). *Bonamia* spp. microcells infect phagocytic hemocytes which are the primary cellular immune defence for oysters (Hine, 1996). *Bonamia* spp. block hemocyte 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 assess intensity of *Bonamia* spp. infection in addition to parasite prevalence to assess oysters for systemic infection, particularly for surveillance in new areas or populations.

Bonamia sp. was first identified in Australia from histology in Port Phillip Bay, Victoria (1991), then in Georges Bay, Tasmania (1992) and in Albany, Western Australia (1993), in all cases in *O. angasi* (see Handlinger et al., 1999; Hine & Jones, 1994). Surveys for *Bonamia* sp. in 1992–93 did not detect *Bonamia* sp. in *O. angasi* in South Australia (SA) (Handlinger et al., 1999). A Pacific oyster health survey identified *Bonamia*-like cells in histopathology in South Australian *C. gigas* in 2003, with no clinical disease, however diagnostic confirmation could not be concluded with histology alone (Diggles, 2003). Lynch et al. (2010) recorded *Bonamia* sp. in *C. gigas* from Spain and Ireland using polymerase chain reaction (PCR), histology and *in situ* hybridisation (ISH). A pilot survey in 2015–2016 (unpublished data, confirmed by the Australian Animal Health

Laboratory and reported to the Office International des Epizooties, OIE) of *O. angasi* in South Australia using quantitative polymerase chain reaction (qPCR) and histology detected *Bonamia exitiosa* Hine, Cochennac, & Berthe, 2001. Due to between-study differences in testing approaches, along with a limited quantitative understanding of the performance of these tests, comparing *Bonamia* spp. prevalence between studies and sites has been problematic.

The overall utility of a test depends on the purpose of the testing and the diagnostic sensitivity (DSe) and specificity (DSp). We interpreted the DSe and DSp of multiple tests using one of two decision rules following Weinstein, Obuchowski and Lieber (2005):

AND-rule: If both tests are positive, then *B. exitiosa* is present.

OR-rule: If either test is positive, then *B. exitiosa* is present.

The following definitions of sensitivity and specificity were modified from Weinstein et al. (2005) to include covariance:

AND-rule:

$$\text{Sensitivity} = \text{DSe(A)} \times \text{DSe(B)} + \text{covp(AB)}$$

$$\text{Specificity} = \text{DSp(A)} + \text{DSp(B)} - [\text{DSp(A)} \times \text{DSp(B)}] - \text{covn(AB)}$$

OR-rule:

$$\text{Sensitivity} = \text{DSe(A)} + \text{DSe(B)} - [\text{DSe(A)} \times \text{DSe(B)}] - \text{covp(AB)}$$

$$\text{Specificity} = \text{DSp(A)} \times \text{DSp(B)} + \text{covn(AB)}$$

Where A/B are the two diagnostic tests being compared; covp is the covariance between test results in an infected individual; covn is covariance between test results in an uninfected individual.

DSe can be increased by applying more than one test and considering the result as positive if either test is positive (OR-rule), while DSp can be increased by considering the result as positive only when both tests return a positive result (AND-rule), as can be seen from the formulae above (Weinstein et al., 2005).

Measures of test performance that consider DSe and DSp include predictive values (PVs), which also depend on prevalence, and likelihood ratios (LRs) which are independent of prevalence. PVs range from 0 to 1.0 and are further defined as positive or negative (PPV or NPV). Higher PPV values (closest to 1.0) support higher probability of a positive sample having disease, whereas higher NPV values (closest to 1.0) support higher probability of a negative test not having disease (Fegan, 2000). The PVs depend strongly on prevalence; when prevalence increases, the PPV increases and the NPV declines, with the opposite true for decreasing prevalence (Fegan, 2000).

The LRs range from zero to infinity with values close to one highlighting little diagnostic value in the test (Caraguel & Vanderstichel, 2013). The diagnostic value improves for LR the

further away from one, with high LR+ (LR for the infected case) desirable for confirming presence of infection, whereas LR- (LR for the uninfected case) should be close to zero for proving absence of infection.

LRs and PVs are calculated as follows:

$LR+ = DSe / (1 – DSp)$

$LR- = (1 – DSe) / DSp$

PPV

$=TP / (TP + FP)$

$=DSe \times prev / [DSe \times prev + (1 – DSp) \times (1 – prev)]$

NPV

$=TN / (TN + FN)$

$=DSp \times (1 – prev) / [(1 – DSe) \times prev + DSp \times (1 – prev)]$

Where (TP/TN) represents true positives/negatives, (FP/FN) represents false positives/negatives and (prev) represents prevalence.

We tested *O. angasi* from a hatchery and three farm sites in South Australia for *B. exitiosa.* A Bayesian Latent Class Model (LCM) was used to assess DSe and DSp of heart smear, qPCR and histology applied singly 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 South Australian *O. 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.

2.3 Methods

2.3.1 Experimental animals and location

Ostrea angasi were sourced from a hatchery and three farm sites in two oyster farming areas in winter 2016. 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) South Australian Aquatic Sciences Centre (SAASC) (West Beach, Adelaide, South Australia) Mollusc Hatchery.

2.3.2 Experimental design

Ostrea angasi were sampled following a randomisation plan where 15 oysters were collected per basket, with 10 baskets chosen per farm site or hatchery. 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 for sampling. Oysters were weighed (OHAUS, Scout, General (SPX) Portable Balance Scales, Model # SPX223), 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:shell ratio calculated [meat:shell ratio = (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 (Table 2.1) for heart smear analysis but were assessed by qPCR and histology.

Table 2.1: Size data (mean ± standard deviation), estimated prevalence (calculated by the Bayesian Latent Class Model) 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				Bayesian	Quantitative parasitology (bootstrap 95%)	
Site	**Weight (g) (Mean ± SD)**	**Shell length (mm) (Mean ± SD)**	**Meat:shell ratio (%) (Mean ± SD)**	**n**	**Estimated prevalence (95% credible intervals)** §	**Mean heart smear intensity cell count (confidence intervals)**	**Mean histology intensity cell count (confidence intervals)**
Coffin Bay A	66.05 ± 14.82	71.34 ± 12.25	32.04 ± 7.14	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 ± 10.69	59.96 ± 7.24	45.36 ± 8.98	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 ± 11.67	58.62 ± 9.23	34.89 ± 8.32	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 ± 0.21	16.67 ± 0.17	20.14 ± 4.83	150	n/a	n/a	n/a

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

‡ SD: standard deviation; 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.

2.3.3 Diagnostic sampling: microscopy analyses (heart smear and histology)

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 smeared 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 in 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 with a compound light microscope (Brightfield Olympus BX53) and the number of *B. exitiosa* cells per smear 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 2.2).

Table 2.2: 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 *B. exitiosa* cells present.	No *B. exitiosa* cells present.
1 – Very light infection	One *B. exitiosa* cell observed per heart smear.	One *B. exitiosa* cell observed per slide after extensive searching.
2 – Light infection	Between 2–10 *B. exitiosa* cells present per heart smear.	Between 2–10 *B. exitiosa* cells observed after searching, 1–2 cells per infected hemocyte.
3 – Moderate infection	More than 10 *B. exitiosa* cells per heart smear, but few parasites per hemocyte.	More than 10 *B. exitiosa* cells per slide, infection widespread and diffuse. Only 1–5 cells per hemocyte.
4 – Heavy infection	*Bonamia exitiosa* present in many hemocytes per heart smear, and many parasites per hemocyte.	*Bonamia exitiosa* cells readily observed in one or more tissues, often associated with hemocytosis.
5 – Systemic infection	*Bonamia exitiosa* present in virtually all hemocytes in each heart smear, many parasites in each hemocyte and extracellularly.	*Bonamia exitiosa* cells abundant in all tissues, many per hemocyte and often extracellular. Hemocytosis always present and lesions sometimes observed.

2.3.4 Diagnostic sampling: qPCR

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 was 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') and 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). 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 a statistically significant increase in fluorescence 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.

2.3.5 Statistical analyses

DSe and DSp of the three tests and estimated prevalence for each farm site were calculated using a Bayesian LCM allowing conditional dependence (covariance) between tests, following Lewis and Torgerson (2012). Code is provided in Appendix A.

Hatchery samples were not included in the LCM because *B. exitiosa* was not detected by histology or qPCR in these 150 samples. Estimated prevalence of *B. exitiosa* in the hatchery was therefore estimated in a Bayesian framework using the prevalence R package (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 indeterminate qPCR results 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 reported the mean and 95% credible intervals for each based on 3,000 simulations. Covariances between tests were considered important when 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, Zakariás, & Rózsa, 2005). Mean

intensity 95% bootstrap confidence intervals with 2,000 replicates were used to assess differences in intensity between farms, with overlapping intervals signifying no difference. In addition, generalised linear models (GLMs) 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 $\alpha = 0.05$.

2.4 Results

2.4.1 Comparison of diagnostic tests (DSe, DSp, LRs, PPV, NPV and covariance)

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 2.1, Table 2.3). 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 2.1, Table 2.3).

DSe of test combinations using the OR-rule were higher than the single smear and qPCR, but not higher than histology alone (Figure 2.1, Table 2.3). 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 2.1, Table 2.3).

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 is positive, the other test is 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 is negative, the other test is likely negative) (Table 2.4).

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 for heart smear (1.56, 1.04–2.35) (Figure 2.1, Table 2.3). LR- for histology (0.25, 0.16–0.35) and qPCR (0.34, 0.25–0.43) were lower than for heart smear (0.67, 0.47–0.96) (Figure 2.1, Table 2.3).

All AND-rule test combinations had higher LR+ than the smear/histology (2.02, 1.43–2.89) OR-rule and smear/qPCR (2.04, 1.47–2.88) test combinations and heart smear (1.56, 1.04–2.35) (Figure 2.1, Table 2.3). 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 2.1, Table 2.3).

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 2.2). In particular, the histology/qPCR AND-rule combination had high PPV (>0.85), even at low prevalence (0.25) (Figure 2.2).

NPV was higher for all OR-rule test combinations than all single tests and all AND-rule test combinations (Figure 2.2). In addition to highest NPV for the histology/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 2.2).

The LCM Model–2 had decreased predicted DSe for the qPCR single test compared to Model–1, but Model–2 had little change to other estimated test performance parameters (Table 2.3).

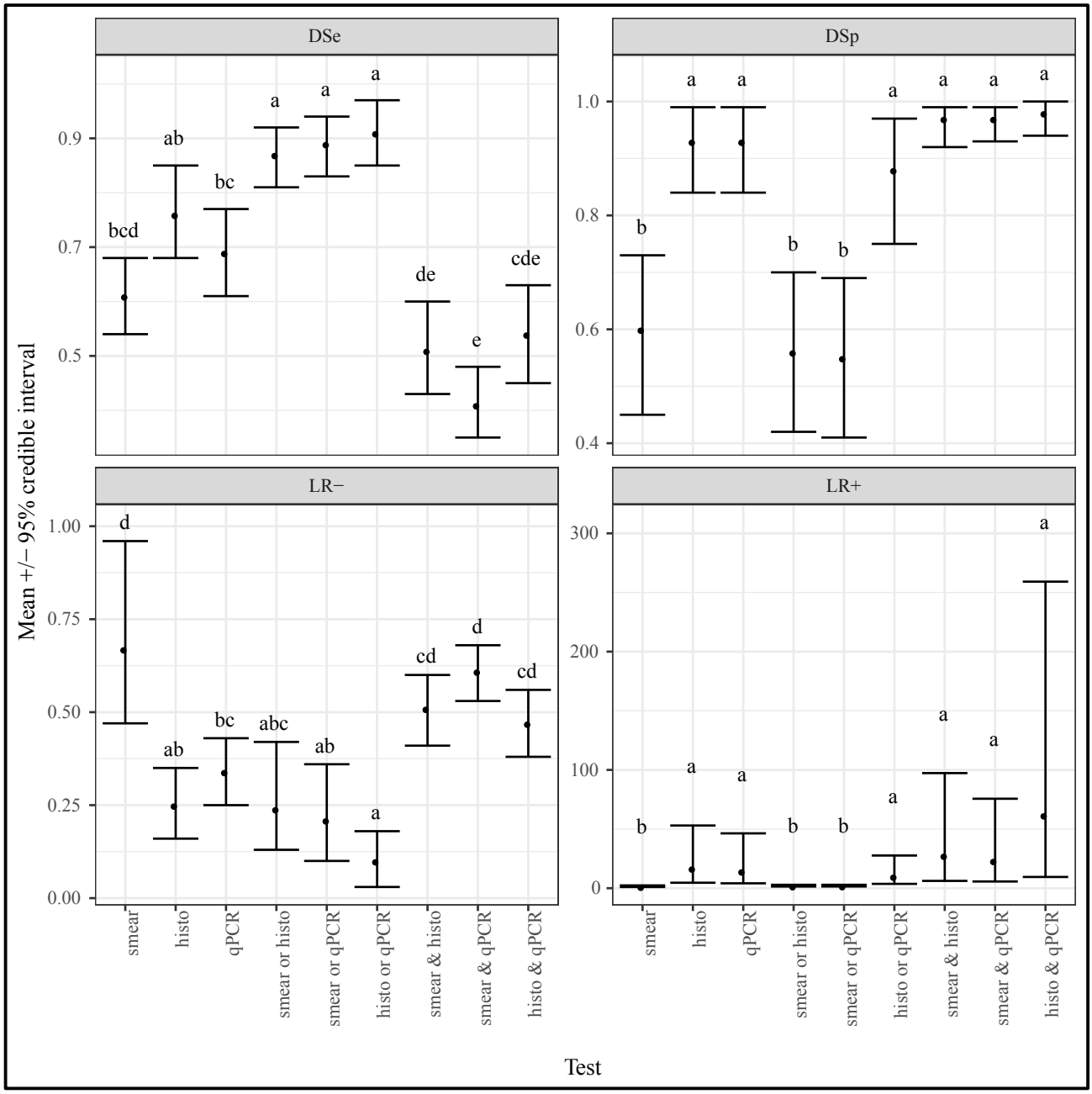

Figure 2.1: Latent Class Model calculated mean and ± 95% credible interval differences for diagnostic sensitivity (DSe) and specificity (DSp) and the likelihood ratio of positive (LR+) and negative (LR-) test results of single and combined diagnostic tests (smear = heart smear; histo = histology; qPCR = quantitative PCR) including both AND-rule (two diagnostic tests, both positive = positive) and OR-rule (two diagnostic tests, either positive = positive) case definitions. Different superscripts denote differences at a 5% level, with a representing the highest value.

Table 2.3: 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.

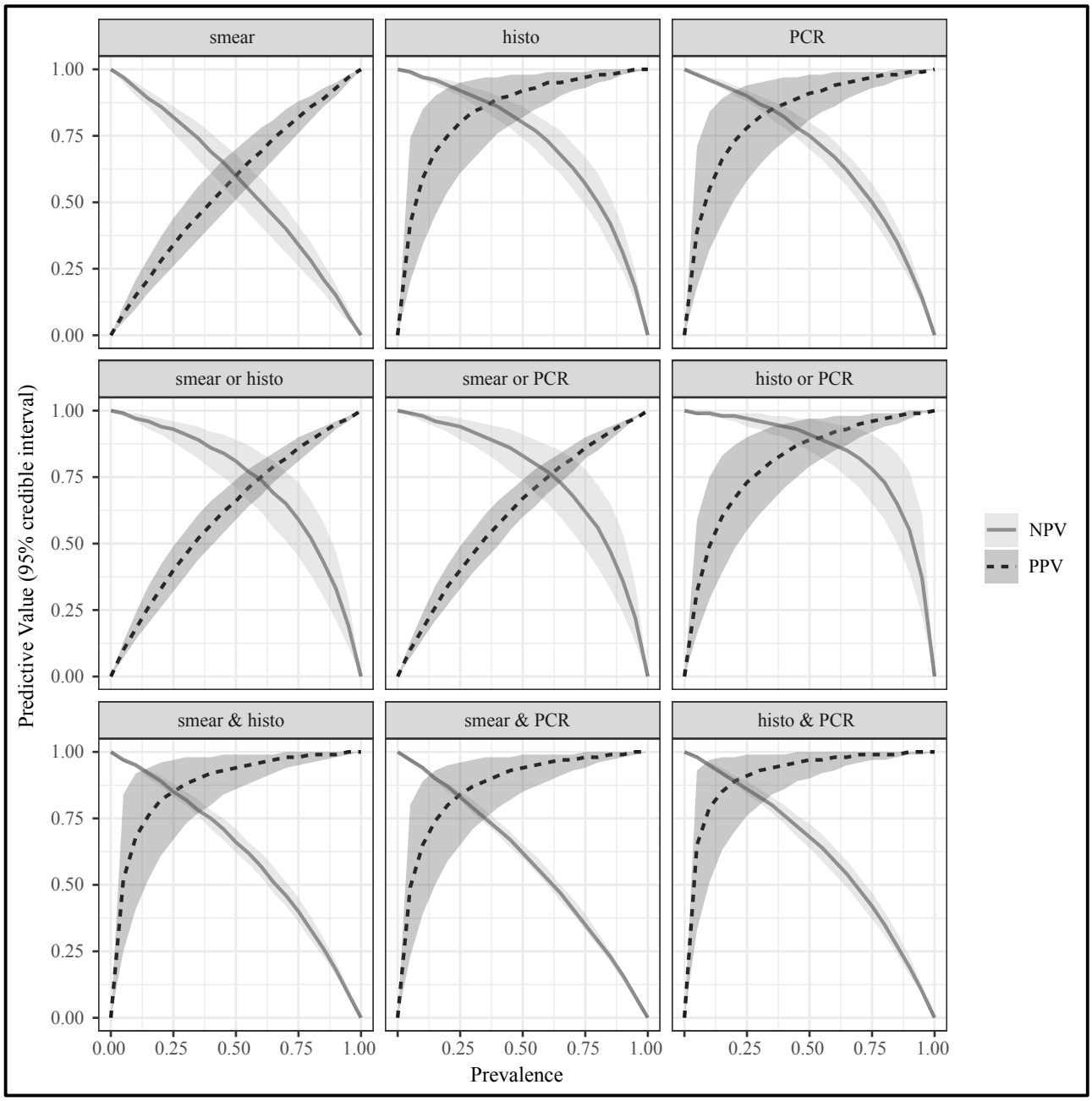

Figure 2.2: Negative Predictive Value (NPV) and Positive Predictive Value (PPV) with 95% credible intervals of single and combined diagnostic tests for *Bonamia exitiosa* with increasing prevalence (smear = heart smear; histo = histology; PCR = quantitative PCR).

Table 2.4: 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) ‡	

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

‡ Different from 0 at a 5% level.

2.4.2 Prevalence

Estimated *B. exitiosa* prevalence for the hatchery, where 150 samples tested negative by both histology and qPCR, was 0.017 (0.000–0.053) (mean, 95% Credible Intervals). For the farm sites, prevalence, as estimated by the LCM, was >0.50 in *O. angasi* (Table 2.1) 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 2.1). *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 2.1).

2.4.3 Intensity

Intensity from histology was highest at Coffin Bay A (mean, bootstrap Confidence Intervals: 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 2.1). *Bonamia exitiosa* intensities from heart smears were numerically higher than intensities from histology for each site, and did not differ between sites (Table 2.1).

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).

2.5 Discussion

We provide the first confirmed record of *B. exitiosa* in South Australia and developed a model to describe DSe and DSp and hence the prevalence in farmed *O. angasi* populations. We also assessed *B. exitiosa* intensity and confirmed that South Australian *O. angasi* are infected at high prevalence, but low intensity. This result is consistent with Corbeil et al. (2009) who noted that Australian *Bonamia* sp. infections in *O. angasi* populations differ from *Bonamia* spp. infections in New Zealand and Europe in that they predominantly display focal lesions with relatively few parasites.

When diagnostic test performance was assessed, histology had higher DSe, NPV and lower LR- than heart smear (Figures 2.1 & 2.2, Table 2.3). This is consistent with OIE (2017). Tests with reliable negative results and high DSe are best for proving freedom of disease (Fegan, 2000). The most sensitive approach for detecting *B. exitiosa* infection has not been unequivocally determined. Diggles et al. (2003) found that heart smear had higher sensitivity than histology for *B. exitiosa* in New Zealand and Lynch, Mulcahy, and 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, Navas, Villalba, & Abollo, 2013; Robert et al., 2009). Unrepresentative negative results will arise if DNA subsampled for qPCR does not contain target DNA (Lynch et al., 2008). The DSe of qPCR is limited by the small physical size of the tissue sample from which DNA is extracted. Localised *B. exitiosa* cell distribution (Diggles et al., 2003) makes sampling an infected section of tissue less likely. 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. We followed Lane, Jones and Poulin (2018) and Lane (2018) and used mantle, gill and the heart tissue remaining from the smear as the tissues for qPCR. Mantle and heart tissue are sites of *B. exitiosa* infection but these tissues do not cause PCR inhibition, unlike digestive gland (Schrader, Shielke, Ellerbroeck, & Johne, 2012).

The DSe of qPCR can be negatively affected by poor sample preparation (Diggles et al., 2003), but we assessed the capacity of the DNA to be amplified, its quality and concentration, and used negative controls at all steps of testing, improving confidence in the quality of the results.

Heart smears gave higher prevalence and reproducibility between laboratories for oysters with high intensity clinical 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 (Table 2.1). In the infected oysters investigated using histology, *B. exitiosa* parasites were concentrated in hemocytes in sinus spaces and connective tissue in the digestive gland and few cells were present in the

hemolymph, gills and mantle indicating that the infection had not become systemic (Corbeil et al., 2009). Lesions associated with *B. exitiosa* were not observed.

Combining any pair of tests with the OR-rule improves DSe and is better for demonstrating disease freedom than smears or qPCR alone (Figure 2.1, Table 2.3). Flannery et al. (2014) and Lynch et al. (2008) also found higher sensitivity for combined tests than single tests for *B. exitiosa.* High DSe contributes to OR-rule combinations having lower LR- and higher NPV (Figures 2.1 & 2.2, Table 2.3) while AND-rule test combinations have lower DSe and higher LR- (Figure 2.1, Table 2.3) 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 budget constraints when designing surveillance plans. Single tests can reduce survey cost, but more expensive OR-rule combinations of tests improve DSe and LR- (Figure 2.1, Table 2.3). 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 (Figures 2.1 & 2.2, Table 2.3), 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 *Bonamia ostreae* Pichot, Comps, Tigé, Grizel, & Rabouin, 1980 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 (Figure 2.1, Table 2.3). 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 (Figures 2.1 & 2.2, Table 2.3). 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 *B. exitiosa* (see OIE, 2017) and results from this study support this (highest DSe and high DSp), although the test is imperfect. Histology has advantages in that it can identify infection intensity for surveying populations and assessing mortalities, provides information on other pathogens, shows indicators of general health and reproductive condition and

provides a permanent record of infection (Diggles et al., 2003). Training diagnosticians to identify *B. exitiosa* in heart smears and histology is, however, more difficult than training for qPCR (Balseiro et al., 2006).

Compared to histology, PCR has the benefit of speed (Marty et al., 2006), but is more costly. PCR can exclude other pathogens that are morphologically similar (Stokes & Burreson, 2001), but targeting DNA limits the ability of PCR to confirm pathogen viability, and it cannot distinguish between infection and disease. When choosing a test, 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 hemocytes (the site of *B. exitiosa* 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 >0.50 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 (see Carnegie et al., 2008; Hine, 1991). While OIE (2017) recommends sampling in January-April based on peak prevalence in *O. chilensis* in New Zealand, peak *B. exitiosa* prevalence can occur in August (Hine, 1991) and 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 from Table 2.1 were classed grade–2–light infection in Table 2.2) in comparison to European farm intensities which were equivalent to our grade–5–systemic infection (Table 2.2) (Culloty, Cronin, & Mulcahy, 2004). *Bonamia exitiosa* 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 Culloty and Mulcahy (1996) although Arzul and 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 *B. exitiosa*. 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 *B. exitiosa* has not been detected.

Differences in *B. exitiosa* prevalence and intensity between farms in South Australia 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 (38 leases) than in Coffin Bay (145 leases) (PIRSA, 2017). Given that *C. gigas* is probably a host of *Bonamia* spp. (see Hill et al., 2014; Lynch et al., 2010), large populations of *C. gigas* may be important reservoirs of *Bonamia* spp. 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 estimated prevalence range estimated by the LCM gives confidence that the hatchery spat were 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 (Table 2.3) is expected because 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 *B. exitiosa* infections in different farming regions can aid regulators and oyster growers to make decisions for development and expansion of oyster farming industries.

Chapter 3 : Rapid transmission of *Bonamia exitiosa* by cohabitation causes mortality in *Ostrea angasi*

Figure 3: *Ostrea angasi* juveniles used for the Chapter 3 cohabitation experiment.

Buss, J. J., Harris, J. O., Tanner, J. E., Wiltshire, K. H., & Deveney, M. R. (2020a). Rapid transmission of *Bonamia exitiosa* by cohabitation causes mortality in *Ostrea angasi*. *Journal of Fish Diseases*, *43*, 227–237, doi: 10.1111/jfd.13116

3.1 Abstract

The haplosporidian *Bonamia* was first detected in Australian shellfish in 1991. Australian isolates in *Ostrea angasi* Sowerby, 1871 were identified as *Bonamia exitiosa* Hine, Cochennac & Berthe, 2001, which threatens development of an *O. angasi* aquaculture industry. European field data suggest that *Bonamia ostreae* Pichot, Comps, Tigé, Grizel & Rabouin, 1980 infections in *Ostrea edulis* Linnaeus, 1758 build slowly but infection dynamics of *B. exitiosa* in *O. angasi* are unknown. We investigated *B. exitiosa* infection in *O. angasi* by cohabiting uninfected juvenile *O. angasi* with adults infected with *B. exitiosa*. Oysters were sampled at 10, 21 and 40 days after cohabitation and *B. exitiosa* prevalence and intensity were assessed. *Bonamia exitiosa* rapidly infected and caused disease in *O. angasi*. Mortalities began at 12 days, with ~50% mortality by day 21 and >85% mortality by day 40. Mortalities displayed pathology consistent with clinical *B. exitiosa* infection. It is likely that time to first infection is influenced by a combination of parasite infectivity, host exposure and host immune capacity. Host death is not required for transmission, but probably facilitates release of parasites from decaying tissue. Understanding *B. exitiosa* transmission informs design and interpretation of field studies and aids development of management strategies for oyster aquaculture.

3.2 Introduction

Native Oysters (*Ostrea angasi* Sowerby, 1871) have been an important resource in Australia since before European settlement, with *O. angasi* being a common food source for coastal Aboriginal people (O'Sullivan, 1980). Commercial fishing of wild *O. angasi* stocks in South Australia began shortly after European colonisation, but the South Australian fishery was closed in 1945 (Olsen, 1994) due to resource depletion caused by overfishing (Alleway & Connell, 2015). An oyster industry has established in South Australia based on farming the introduced Pacific oyster (*Crassostrea gigas* Thunberg, 1793) (see Olsen, 1994). The threat posed to *C. gigas* aquaculture by ostreid herpes virus-1 (OsHV-1) microvariant, however, has increased interest in cultivating *O. angasi*, which are not susceptible to OsHV-1 disease (Kirkland, Hick, & Gu, 2015). Restoration of bivalve reefs has further prompted interest in *O. angasi* cultivation (Gillies, Crawford, & Hancock, 2017). Disease caused by *Bonamia exitiosa* Hine, Cochennac & Berthe, 2001 however, remains a significant hurdle for development of *O. angasi* aquaculture (Nell, 2001; O'Connor & Dove, 2009).

Bonamia spp. are haplosporidian parasites of oysters (Morga et al. 2017; Sierra et al. 2016). *Bonamia* spp. infect the phagocytic hemocytes of oysters, in which *Bonamia* spp. cells spread to host gills, digestive gland and mantle (Sweet & Bateman, 2015). *Bonamia* spp. can infect hosts directly (Arzul & Carnegie, 2015; Culloty et al., 1999; Engelsma, Culloty, Lynch, Arzul, & Carnegie, 2014), but the mechanisms of infection and parasite release are poorly described. Host death may facilitate release of infective cells (Arzul & Carnegie, 2015; Hine, 1996; Hine & Jones, 1994), and consumption of cells when filter feeding is a likely mode of infection (Hine & Jones, 1994; Flannery, Lynch, & Culloty, 2016). Direct transmission means that farming with high oyster densities creates an environment that is favourable for transmission and parasitaemia (Owens, 2012).

Parasite-host interactions of *Bonamia* spp. have been investigated worldwide including susceptibility and genetic resistance to *B. ostreae* of farmed *O. edulis* populations (Martin, Gérard, Cochennec, & Langlade, 1993; Montes, Ferro-Soto, Conchas, & Guerra, 2003), age-related susceptibility of farmed (Arzul et al., 2011; Culloty & Mulcahy, 1996) and wild *O. edulis* to *B. ostreae* infection (Lallias et al., 2008), *B. ostreae* bivalve host range (Culloty et al., 1999), environmental influences of *B. exitiosa* infection in *Ostrea chilensis* Küster, 1844 wild populations (Hine, Diggles, Parsons, Pringle, & Bull, 2002) and *B. exitiosa* dynamics in farmed *Crassostrea ariakensis* Fujita, 1913 populations (Audemard, Carnegie, Bishop, Peterson, & Burreson, 2008a; Audemard et al., 2008b).

CHAPTER 3: COHABITATION

Bonamia spp. occur in both hemispheres (Carnegie & Engelsma, 2014) and a *Bonamia* sp. infection was first reported in Australia in *O. angasi* in 1991 (Hine & Jones, 1994). Australian *Bonamia* sp. infection is usually associated with poor oyster condition, but infection can also occur in oysters which appear healthy. Focal lesions from *Bonamia* sp. are most common within *O. angasi* digestive gland and gills and are less common in *O. angasi* mantle and gonad. Systemic clinical infection in *O. angasi* is rare, except for populations that are showing mortalities (Corbeil, Handlinger, & Crane 2009). Within Australia, clinical disease and mortality due to *Bonamia* sp. have been recorded in *O. angasi* in Victoria and Western Australia, and *Bonamia* sp. infection has been confirmed in *O. angasi* in Tasmania and New South Wales (Corbeil et al., 2009). A *Bonamia* sp. was identified in *O. angasi* in South Australia by Buss, Wiltshire, Prowse, Harris, and Deveney (2019, Chapter 2). Extensive investigations, including genome sequencing of a Victorian isolate, have identified all southern Australian isolates of *Bonamia* from *O. angasi* as *B. exitiosa* (see Bradley, 2019).

Bonamia exitiosa infection dynamics in *O. angasi* are unknown. We aimed to begin to understand *B. exitiosa* infection in *O. angasi* by determining time to first infection, and prevalence, intensity and mortality over time in a laboratory cohabitation trial.

3.3 Methods

3.3.1 Experimental animals

Juvenile *O. angasi* were sourced from the South Australian Research and Development Institute (SARDI) South Australian Aquatic Sciences Centre (SAASC) Mollusc Hatchery (West Beach, Adelaide, South Australia). Juvenile oysters were tested using real-time PCR (Corbeil et al., 2006) (n = 150) and histology (n = 150), which did not detect *B. exitiosa* (mean Bayesian estimated prevalence, 95% credible intervals: 0.017, 0.000–0.05, Buss et al., 2019, Chapter 2). Adult *O. angasi* were collected from Coffin Bay, South Australia from a site shown to have 0.90 (0.78–0.99) *B. exitiosa* prevalence (Buss et al., 2019, Chapter 2 mean Bayesian estimated prevalence, 95% credible intervals). Oysters were maintained separately in floating baskets at the South Australian Aquatic Biosecurity Centre (SAABC), Roseworthy Campus, South Australia, in 500 L fibreglass tanks with aeration and a canister filter (Aquaone Nautilus 2700UVC) until use in experiments.

3.3.2 Experimental system and design

The experimental system comprised eight 52 L plastic tanks containing aerated seawater. Every two to three days the water was exchanged. Oysters were fed 1.25 L (2.0 x 10^6 cells/mL) of a mixed culture of *Chaetoceros muelleri* Lemmermann, 1898, *Skeletonema costatum* (Greville) Cleve, 1873 and *Pavlova lutheri* (Droop) Green, 1975 per tank following water exchange. Tank placement, maintenance and operation of the system were designed to prevent cross-contamination. Water quality was within normal parameters in all tanks for the duration of the experiment: water temperature was maintained at 16.83 ± 2.27°C (mean ± SD), salinity was maintained at 38 psu and dissolved oxygen was 97.88 ± 1.53% or 7.74 ± 0.40 mg/L (mean ± SD). Temperature and salinity ranges for recipients were based on autumn or spring oceanographic data for Coffin Bay (see Kämpf & Ellis, 2015).

2400 juvenile *O. angasi* (weight: 1.61 ± 0.81 g, shell length: 22.75 ± 4.54 mm, 14 months old) (mean ± SD) were randomly assigned to eight tanks (n = 300 per tank). 40 adult (donor) *O. angasi* (weight: 72.03 ± 15.02 g, shell length: 71.97 ± 3.76 mm) (n = 10 donors per tank) were assigned to four of the tanks as a source of *B. exitiosa* infection with juvenile recipients. The remaining four tanks held juvenile controls without donors. On day 10, 21 and 40 post-cohabitation, 44 recipients and 44 controls were sampled for heart smear and histology (Table 3.1). Mortalities were preferentially selected for collection on sampling days to assess if oysters were dying due to *B. exitiosa.* Samples with highest heart smear score were chosen for histology. 10 samples were tested for both histology and heart smear for every exposure and time treatment (Table 3.1). All

donor animals remaining in the exposure tanks were collected at the end of the experiment (Table 3.1). All live donors were sampled for heart smear and 10 selected for histology (Table 3.1).

Oysters (controls, recipients and donors) were inspected every two to three days and mortalities that occurred on non-dedicated sampling days were removed, weighed, measured and sampled for diagnostic testing (see Table 3.1 for sample numbers). All mortalities were sampled for heart smear (Table 3.1). From these mortalities, 21 oysters (recipients and donors) with highest heart smear intensities were also sampled for histology (Table 3.1). All oysters that were collected were replaced with oysters in labelled mesh pouches separate to the experimental animals to maintain tank biomass.

Table 3.1: Total number of live and dead recipient/control and donor oysters sampled for heart smear and histology on each dedicated sampling day (days 10, 21 and 40) and mortalities that were sampled in the periods between these: days 0–10, 11–21 or 22–40.

			Number of oysters sampled for heart smear and/or (histology) †					
			Sampling days			**Mortalities sampled during experiment** §		
Oyster code	**Oyster age**	**Oyster status**	**Day 10**	**Day 21**	**Day 40**	**Days 0–10**	**Days 10–21**	**Days 22–40**
Recipient	Juvenile	Live	**44** (10)	**0**	**44** (10)	n/a	n/a	n/a
Recipient	Juvenile	Dead	**0**	**44** (10)	**0**	**0**	**65** (7)	**98** (6)
Control	Juvenile	Live	**44** (10)	**43** (9)	**44** (10)	n/a	n/a	n/a
Control	Juvenile	Dead	0	**1** (1)	**0**	**0**	**0**	**0**
Donor	Adult	Live	**0**	**0**	**30** ‡ (10)	n/a	n/a	n/a
Donor	Adult	Dead	**0**	**0**	**0**	**1** (1)	**3** (3)	**7** (4)

† Number of oysters sampled for heart smear are indicated in bold, number of oysters sampled for histology and heart smear are within parentheses.

‡ All remaining donor oysters were sampled only on day 40.

§ n/a: Not applicable.

3.3.3 Diagnostic sampling: heart smear and histology

Histology and heart smears were prepared as described by Buss et al. (2019, Chapter 2). Histology and heart smears were examined with a compound light microscope (Brightfield Olympus BX53) with *B. exitiosa* cell intensity graded using the scale in Buss et al. (2019, Chapter 2).

3.3.4 Terms and statistical analyses

Parasitology terms are consistent with Bush, Lafferty, Lotz and Shostak (1997).

Survival of recipient and control oysters was assessed over 40 days using Kaplan-Meier analysis with Log-Rank and Breslow tests in IBM SPSS version 23 for Macintosh (IBM SPSS Inc., Chicago, IL).

Intensity of *B. exitiosa* from histology and heart smears were analysed using Quantitative Parasitology version 1.0.14 (Reiczigel, Marozzi, Fábián, & Rózsa, 2019) using 95% bootstrap confidence intervals for mean intensities with 2,000 replicates. Data were assessed as being different when confidence intervals did not overlap. Intensity data used the subset of oysters that were tested by both histology and heart smear.

A Bayesian Latent Class Model (LCM) was used to calculate estimated prevalence with credible intervals in all time periods using JAGS code modified from the prevalence R package (Devleesschauwer et al., 2015) to allow simultaneous estimation of prevalence for multiple treatments and time points. This model used results from oysters tested by both histology and heart smear. Markov Chain Monte Carlo (MCMC) simulations were obtained by running the model in JAGS v. 4.3.0 (Plummer, 2017) using three chains for 10,000 iterations, thinned at a rate of 10, 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 using the Gelman-Rubin convergence statistic, and confirmed by visual inspection of trace, density and autocorrelation plots generated using the MCMCvis package (Youngflesh, 2018). Posterior predictions of diagnostic sensitivity (DSe), diagnostic specificity (DSp) and conditional covariance for positive or negative disease status from Buss et al. (2019, Chapter 2) were used to inform priors for the latent class model. Beta prior parameters used for DSe and DSp for heart smear and histology are specified in Table 3.2. These beta priors reflected 95% confidence that each of these parameters fall within the credible interval with the mean specified in Table 3.2.

Generalised Linear Models (GLMs) were used to separately assess patterns in histology and heart smear intensity to account for different samples sizes (Table 3.1). Analyses of heart smear intensity included data from the oysters that were not tested by histology. Due to the control oysters being *B. exitiosa* negative across sampling times, analyses of intensity were performed only for recipient and donor oysters. GLM compared intensities from histology in live recipient oysters between day 10 and 40; day 21 was excluded as all sampled recipients were mortalities on this day (Table 3.1). GLM also compared intensities from heart smear between recipient and donor oysters; data from mortalities and live oysters collected in the time period day 22–40 were included to test the effect of status (dead or alive) as well as age (adult donor or juvenile recipient). GLM was also used to compare intensities from heart smear in live and dead recipients in the time periods day 0–10 and day 22–40 to see the effect of status and time; the time period day 11–21 was excluded as all recipients sampled were mortalities in this period.

A negative binomial distribution was used for all intensity analyses, due to over dispersion of data relative to a Poisson distribution. Negative binomial GLMs used the MASS package (Venables & Ripley, 2002).

For all analyses, time periods included live oysters and mortalities that were sampled on the dedicated sampling day at the end of that period and mortalities that occurred subsequent to the previous dedicated sampling day.

Table 3.2: The beta priors, associated mean and 95% credible intervals for diagnostic sensitivity (DSe) and diagnostic specificity (DSp) for heart smear and histology, used to calculate estimated prevalence, plus the posterior predictions for DSe and DSp of each test. †

Test	DSp or DSe	Beta priors	Mean, 95% credible interval	Posterior predications (mean, credible interval)
Heart smear	**DSe**	(113, 72)	0.61 (0.54–0.68)	0.70 (0.64–0.72)
Heart smear	**DSp**	(27, 18)	0.60 (0.45–0.73)	0.68 (0.57–0.72)
Histology	**DSe**	(75, 23)	0.76 (0.68–0.85)	0.82 (0.74–0.84)
Histology	**DSp**	(40, 3)	0.93 (0.84–0.99)	0.95 (0.90–0.97)

† All priors were derived from Buss et al. (2019, Chapter 2)

3.4 Results

3.4.1 Survival

There was a significant decrease in survival of recipient oysters compared to control oysters at day 40 ($p < 0.001$ Kaplan-Meier, Figure 3.1). One control oyster died over the duration of the experiment. At day 40, survival of recipient oysters was 12.43% while survival of control oysters was 99.9% (Figure 3.1). The first recipient oyster mortality occurred on day 12 and by day 21, survival decreased to 44.8–54.8% (Figure 3.1). Because mortalities were sampled preferentially, during the mortality event on day 21, all recipient oysters sampled on day 21 were mortalities (see Table 3.1).

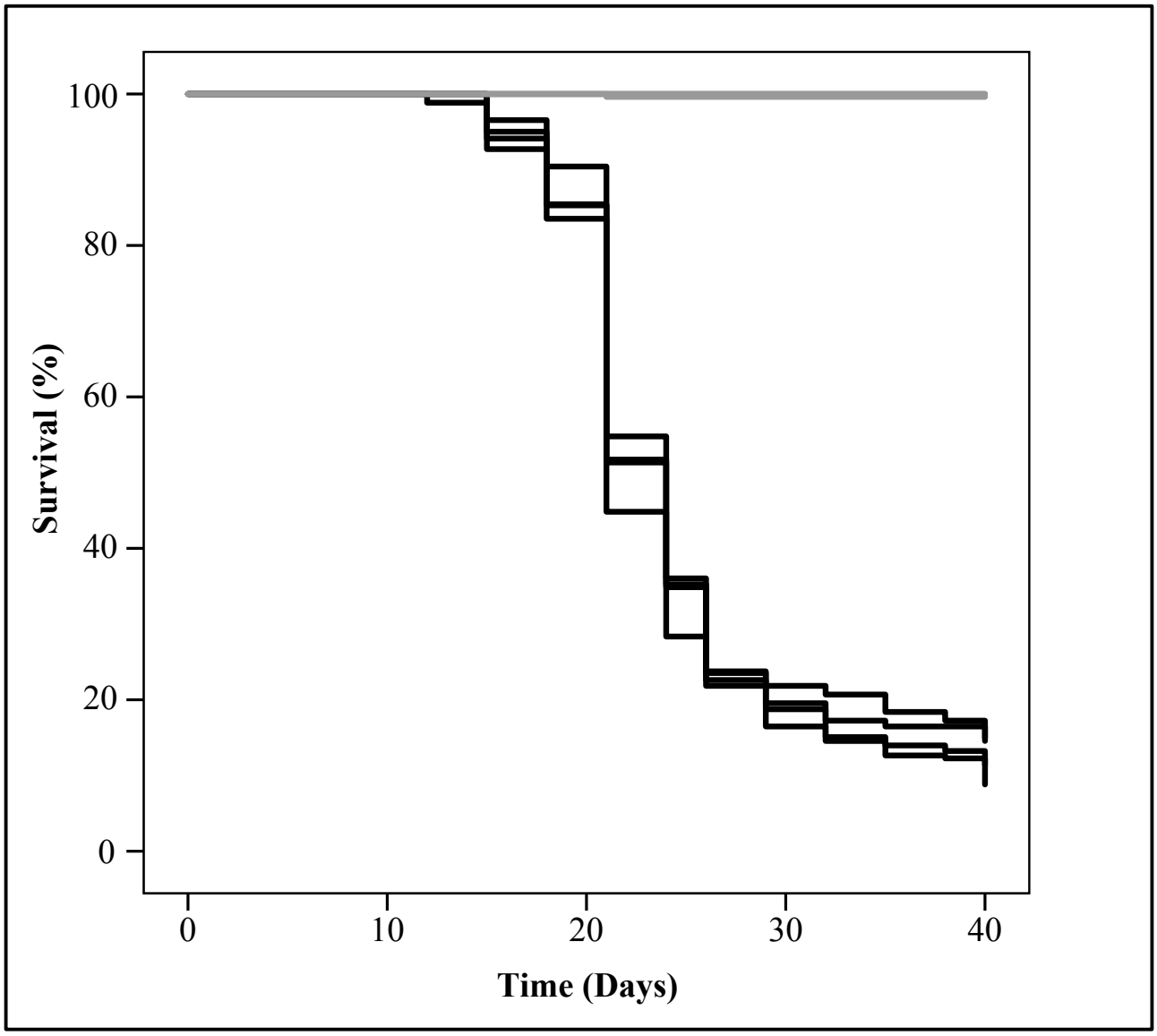

Figure 3.1: Kaplan-Meier survival curve for *Ostrea angasi* juveniles in recipient *Bonamia exitiosa* tanks (black lines) and control tanks (grey lines) for 40 days. N = 300 for each line. Survival for all control tanks > recipient tanks, $p < 0.05$.

3.4.2 Prevalence and diagnostic performance

Estimated *B. exitiosa* prevalence increased over time, with higher estimated prevalence for recipient oysters sampled in the time periods days 11–21 or days 22–40, than recipient oysters sampled in the time period 0–10 or control oysters sampled in any time period (Table 3.3). Donors had higher estimated prevalence than recipients sampled in the time period 0–10 or controls sampled in any time period (Table 3.3).

The posterior predicted mean DSe and DSp were higher than the means from Buss et al. (2019, Chapter 2) used as priors in the Bayesian LCM (Table 3.2) but their 95% credible intervals overlapped, indicating that DSe and DSp for histology and heart smear were similar between this study and Buss et al. (2019, Chapter 2).

Table 3.3: Size data, apparent prevalence, Bayesian estimated prevalence with credible intervals and mean intensity with confidence intervals (calculated through Quantitative Parasitology) of *Bonamia exitiosa* in *Ostrea angasi* from heart smears and histology sampled in the time periods: days 0–10, 11–21 or 22–40. Confidence intervals could not be calculated when values were constant. ‡

		Size data					Bayesian † § ¶	Quantitative Parasitology (bootstrap, 95%) †	
Treatment sampling periods	**Treatment exposure** ‡	**Weight (g) (Mean ± SD)**	**Shell length (mm) (Mean ± SD)**	**Meat:shell ratio (%) (Mean ± SD)**	**n**	**Apparent prevalence** §	**Estimated prevalence (95% credible intervals)**	**Mean heart smear intensity cell count (confidence intervals)**	**Mean histology intensity cell count (confidence intervals)**
Days 0–10	**Control**	1.54 ± 0.63	22.98 ± 4.78	22.70 ± 5.21	10	0	0.11 (0.00–0.15) [c]	0	0
Days 11–21	**Control**	1.74 ± 0.94	23.69 ± 5.16	26.53 ± 7.77	10	0	0.11 (0.00–0.15) [c]	0	0
Days 22–40	**Control**	1.89 ± 0.55	25.57 ± 2.15	29.49 ± 5.17	10	0	0.11 (0.00–0.15) [c]	1.50 (1.00–1.55) [c]	0
Days 0–10	**Recipient**	1.32 ± 0.88	20.61 ± 4.75	25.06 ± 8.30	10	0.40	0.51 (0.18–0.64) [b]	5.30 (4.00–6.90) [b]	5.50 (2.75–8.00) [b]
Days 11–21	**Recipient**	1.12 ± 0.41	21.71 ± 3.38	13.75 ± 5.13	17	0.94	0.93 (0.78–0.98) [a]	15.40 (11.40–20.30) [a]	19.20 (15.00–23.40) [a]
Days 22–40	**Recipient**	1.70 ± 0.95	22.75 ± 5.76	23.43 ± 11.86	16	1.00	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 ± 16.52	74.65 ± 4.75	60.50 ± 12.80	18	1.00	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 recipient treatments included alive and dead juveniles sampled per time period and only included oysters that were assessed for two tests (both histology and heart smear). The donor treatment included live adults sampled on day 40 and any adult mortalities that occurred throughout the trial.

§ 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. (2019, Chapter 2). SD: standard deviation; n: sample number per treatment/time period.

3.4.3 Intensity

Recipient oysters sampled in the period day 0–10 had lower *B. exitiosa* intensity from both heart smears and histology than recipient oysters sampled in the periods day 11–21, day 22–40 or donor oysters (Table 3.3). Two control oysters contained cells in heart smears that were identified as likely to be *B. exitiosa*, but these animals were both negative by histology and using the AND-rule to maximise DSp for prevalence, were classified as negative.

GLM showed live recipient oysters had significantly higher *B. exitiosa* histology intensities on day 40 than on day 10 (LRT: χ^2 (1) = 22.38, $p < 0.001$) (Figure 3.2). Donor oysters (dead and alive) sampled in the period day 22–40 had significantly higher *B. exitiosa* intensities from heart smears than recipient oysters (dead and alive) from the same time period (LRT: χ^2 (1) = 166.65, $p < 0.001$) (Figure 3.2). Recipient oysters (dead and alive) sampled in the period day 22–40 had significantly higher intensities from heart smear than recipients sampled in the period day 0–10 (LRT: χ^2 (2) = 7.15, $p < 0.001$) (Figure 3.2). There was no significant difference in heart smear intensity between dead and live recipient oysters (LRT: χ^2 (1) = 0.511, $p = 0.475$). Mean *B. exitiosa* intensities from heart smear and histology, for donors and recipients per time period are summarised in Figure 3.2.

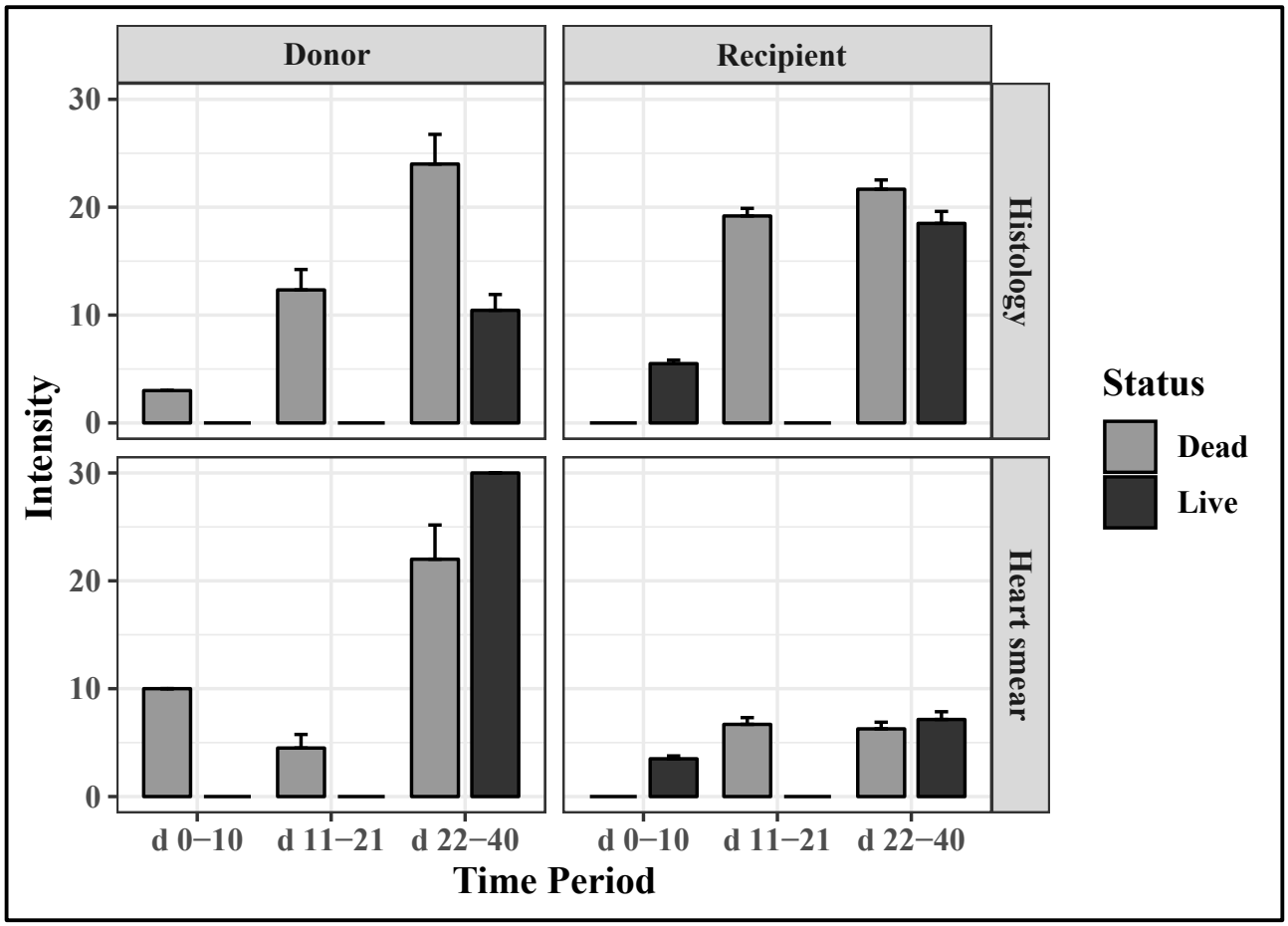

Figure 3.2: Mean *Bonamia exitiosa* intensity from heart smear and histology for dead and alive donor and recipient *Ostrea angasi* sampled in the time periods: day 0–10, day 11–21 and day 22–40. Mean ± SE. Recipients: n = 295 for heart smear, n = 43 for histology; Donors: n = 40 for heart smear, n = 18 for histology.

3.4.4 Pathology

In donor and recipient oysters, *B. exitiosa* cells were observed in the gill, mantle and gonad and particularly in the connective tissue and sinus spaces of the digestive gland (Figure 3.3). Intracellular and extracellular infection was observed and concentrated aggregations of *B. exitiosa* cells were common (Figures 3.3 & 3.4). Few lesions were observed in this study. For recipient oysters after 11 days of exposure grade–3 infections (moderate) were most common in heart smear and histology, but grade–4 (heavy) and grade–5 (systemic) infections were also observed (Table 3.4). Donors varied in heart smear and histology infection from grade–2 (light) to grade–5 (systemic) infection (Table 3.4).

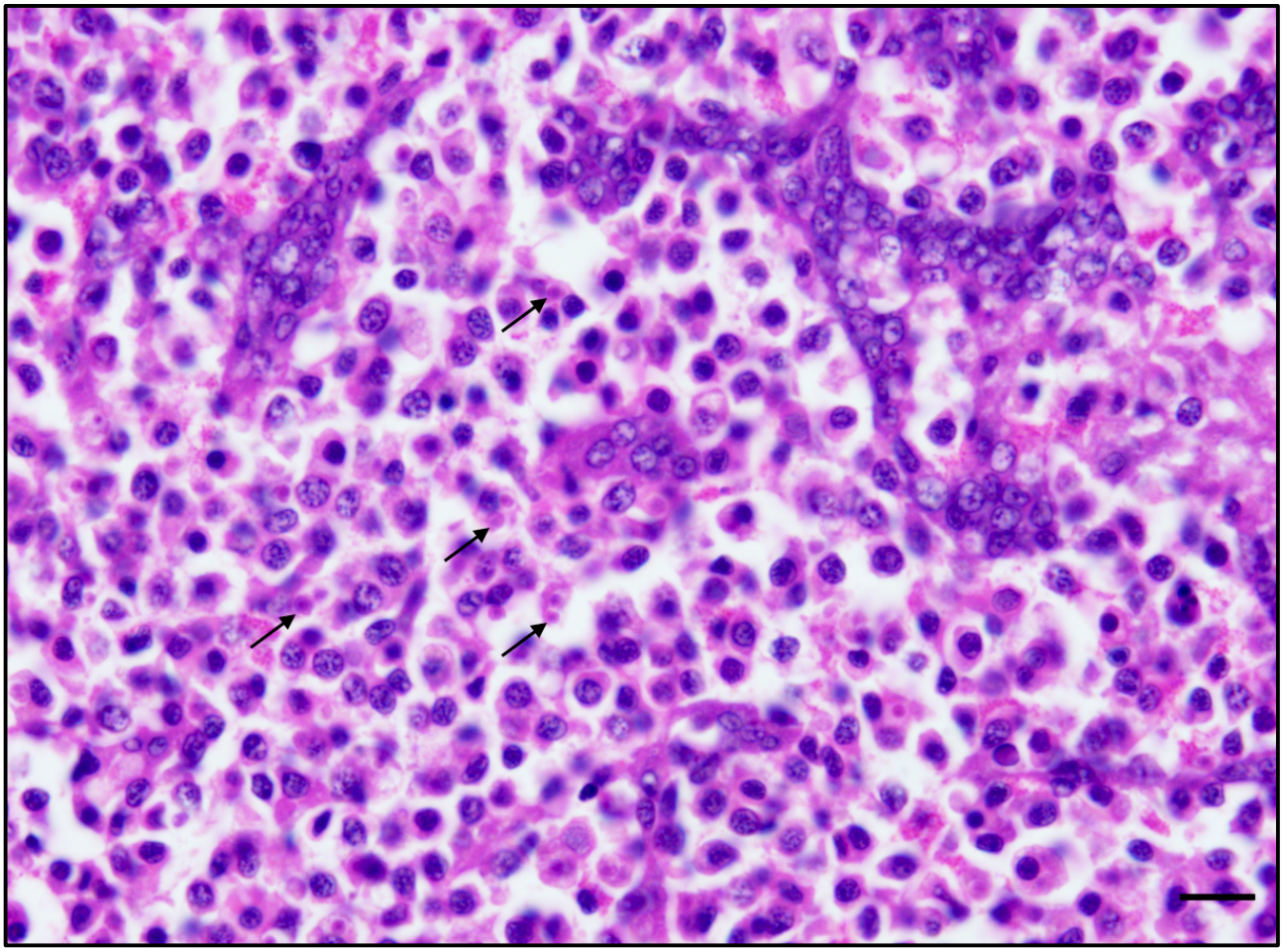

Figure 3.3: Histological section of a recipient *Ostrea angasi* with a heavy-grade-4 *Bonamia exitiosa* infection (see Buss et al., 2019, Chapter 2) sampled on day 21. Arrows point to *B. exitiosa* cells infecting hemocytes in the connective tissue and sinus spaces in the digestive gland. Hematoxylin and eosin stain. Scale bar represents 10 µm.

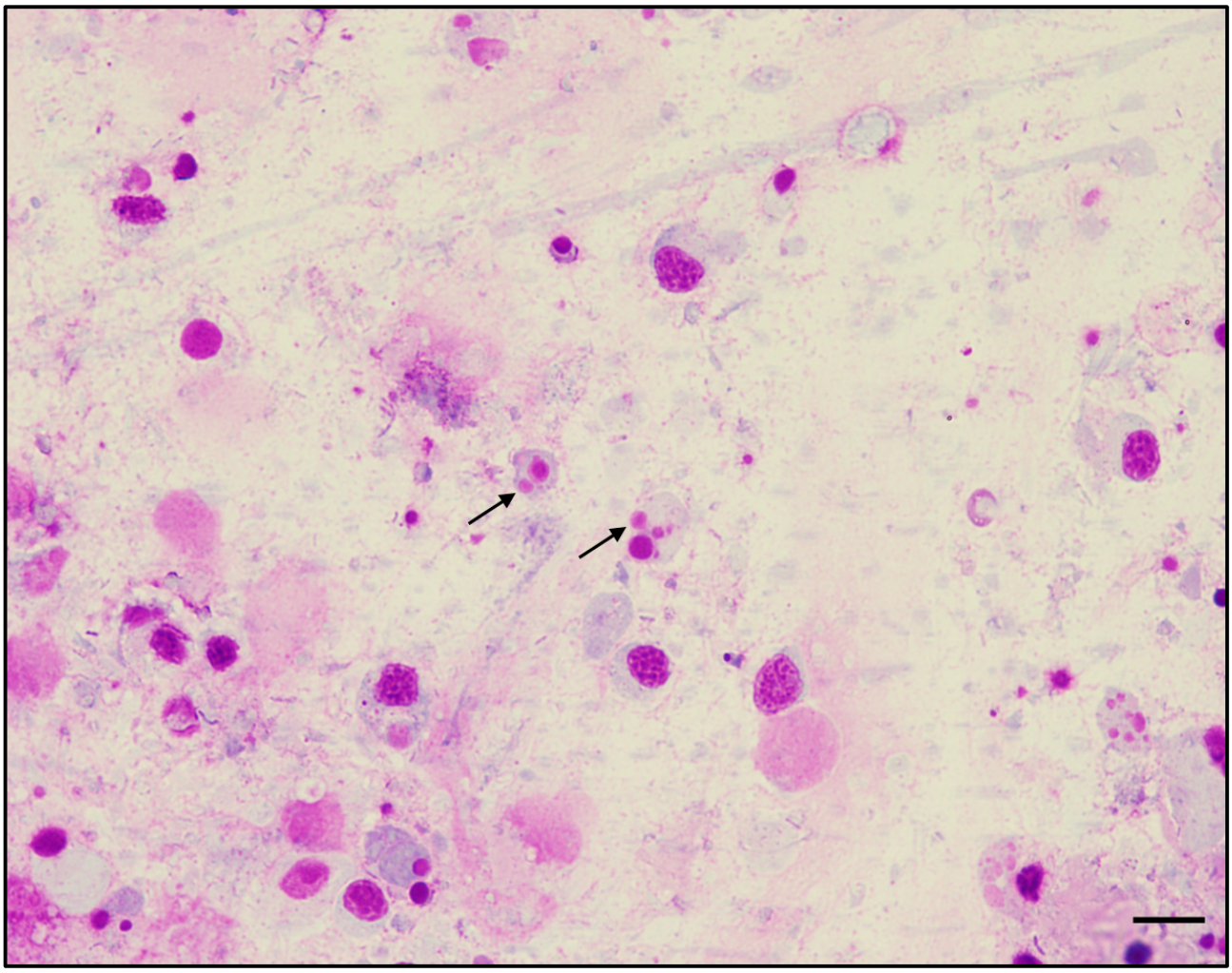

Figure 3.4: Heart smear of a recipient *Ostrea angasi* with moderate-grade-3 *Bonamia exitiosa* infection (see Buss et al., 2019, Chapter 2) sampled on day 21. Arrows point to *B. exitiosa* cells within hemocytes. Hemacolor® stain. Scale bar represents 10 µm.

Table 3.4: Grading of *Bonamia exitiosa* infection in *Ostrea angasi* by heart smear and histology sampled in the time periods: days 0–10, 11–21 or 22–40.

			Heart smear grade of infection (n) ‡						Histology grade of infection (n) ‡					
Treatment sampling periods	**Treatment exposure †**	**n**	**0**	**1**	**2**	**3**	**4**	**5**	**0**	**1**	**2**	**3**	**4**	**5**
Days 0–10	**Control**	**10**	10	0	0	0	0	0	10	0	0	0	0	0
Days 11–21	**Control**	**10**	10	0	0	0	0	0	10	0	0	0	0	0
Days 22–40	**Control**	**10**	8	1	1	0	0	0	10	0	0	0	0	0
Days 0–10	**Recipient**	**10**	0	0	10	0	0	0	6	0	4	0	0	0
Days 11–21	**Recipient**	**17**	0	0	7	9	1	0	1	0	3	8	4	1
Days 22–40	**Recipient**	**16**	0	0	6	9	1	0	0	0	2	12	1	1
Days 0–40	**Donor**	**18**	0	0	4	7	7	0	0	0	8	8	0	2

† Control and recipient treatments included alive and dead juveniles sampled per time period. The donor treatment included live adults sampled on day 40 and any adult mortalities that occurred throughout the trial. n: sample number per treatment/time period.

‡ Grading system is described in Buss et al. (2019, Chapter 2): grade 0: not infected, grade 1: very light infection, grade 2: light infection, grade 3: moderate infection, grade 4: heavy infection, grade 5: systemic infection.

3.5 Discussion

Ostrea angasi became infected rapidly after exposure to *B. exitiosa*, with estimated prevalence reaching >0.5 by day 10 and >0.9 by day 40 (Table 3.3). Transmission of *B. exitiosa* in *C. ariakensis* also occurred rapidly in Bogue Sound, USA, but apparent prevalence after 14 days field exposure was 0.03, and at 21 days was 0.3 (Audemard, Carnegie, Hill, Peterson, & Burreson, 2014). Our infection model places high infection pressure on recipient oysters by confining them with several large donors in a small static system, whereas Audemard et al. (2014) exposed their recipients to water from an infected estuary with much higher volume and flow which is likely to provide a lower infection pressure. In the northern hemisphere, time to first *B. ostreae* infection in *O. edulis* is >2 months in field exposures in enzootic areas and in lab trials the first *B. ostreae* mortalities occurred after >4 months cohabitation (Lallias et al., 2008) (Table 3.5). Our data suggest that time to first infection in recipients reflects complicated influences of extrinsic factors and innate aspects of the parasite-host system.

Time to first infection and changes in prevalence and intensity of recipients are influenced by the immunocompetence of the individual hosts and the average immunological capacity of the host population. Gervais, Chollet, Renault and Arzul (2016) and Comesaña et al. (2012) found that *O. edulis* hemocytes are more susceptible to *B. ostreae* infection than *C. gigas* hemocytes. The short time from recipient *O. angasi* exposure to first *B. exitiosa* infection may be caused by *O. angasi* having a lesser capacity to mount an effective immune response against *B. exitiosa* than other oyster species. Oysters lack immune memory homologous to vertebrates (Wang, Song, & Song, 2018) but display immune priming after exposure to a pathogen (Contreras-Garduño et al., 2016; Little & Kraaijeveld, 2004) and the offspring of primed individuals can display increased immune capacity (Green & Speck, 2018). *Ostrea edulis* from *B. ostreae* endemic areas are less susceptible to *B. ostreae* than *O. edulis* from *B. ostreae* free areas (Culloty, Cronin, & Mulcahy, 2004), but it is unclear if this was due to immune priming or mass selection in wild populations for immune competence when exposed to *B. ostreae*. The parents of our recipient animals were from farms in Coffin Bay, where *B. exitiosa* occurs at high prevalence (Buss et al., 2019, Chapter 2), but our *O. angasi* recipients tested negative for *B. exitiosa* and appear to have been naïve to infection. The first detection of a *Bonamia* sp. in South Australia occurred in 2003 in *C. gigas* (see Diggles, 2003) and the first confirmed *B. exitiosa* infections were reported in 2015 (Bradley, 2019, Buss et al. 2019, Chapter 2). Oysters in South Australia have therefore not had been exposed over a sufficient number of generations to experience mass selection for improved capacity to resist *B. exitiosa* infection. The susceptibility of the oysters we tested suggests that the parents of our recipient stock had not evolved a heritable capacity to resist *B. exitiosa* infection and/or that offspring of

individuals primed by exposure to *B. exitiosa* may not display increased capacity to mount an immune response to *B. exitiosa* challenge.

Table 3.5: The time to first *Bonamia exitiosa* or *B. ostreae* infection and time to first mortality, positive for *Bonamia* spp. infection in *Ostrea edulis* and *Crassostrea ariakensis* cohabitation trials.

Parasite	Host	Factor	Time	Experiment location	Notes	Reference
B. ostreae	*O. edulis*	Time to first infection	6 months	Cork Harbour, Ireland	Field trial, cohabitation	(Culloty & Mulcahy, 1996)
B. ostreae	*O. edulis*	Time to first infection	2–4 months	Cork Harbour and Galway Bay, Ireland	Field trial, cohabitation	(Lynch, Armitage, Wylde, Mulcahy, & Culloty, 2005)
B. ostreae	*O. edulis*	Time to first infection	3–6 months	Arosa, Aldan and Vigo estuaries, Galicia, Spain	Field trial, cohabitation	(Montes, 1991)
B. ostreae	*O. edulis*	Time to first infection	12–24 months	Cambados and Bueu, Galicia, Spain	Field trial, cohabitation	(Montes, Ferro-Soto, Conchas, & Guerra, 2003)
B. ostreae	*O. edulis*	Time to first positive mortality	7 months	Western North America	Lab trial, cohabitation Recipient: Donor ratio was 1:1	(Elston, Farley, & Kent, 1986)
B. ostreae	*O. edulis*	Time to first positive mortality	4 months	La Tremblade, France	Lab trial, cohabitation Recipient: Donor ratio was 50:21	(Lallias et al., 2008)
B. exitiosa	*C. ariakensis*	Time to first infection	3–4 weeks	Bogue Sound & Masonboro Sound, North Carolina, United States of America (USA)	Field trial, cohabitation	(Carnegie et al., 2008)
B. exitiosa	*C. ariakensis*	Time to first infection	28 days	Virginia Institute of Marine Science (VIMS), Virginia, USA	Lab trial, cohabitation Recipient: Donor ratio was 1:1	(Audemard, Carnegie, Hill, Peterson, & Burreson, 2014)
B. exitiosa	*C. ariakensis*	Time to first infection	14 days	Bogue Sound, North Carolina, USA	Field trial, cohabitation	(Audemard et al., 2014)

In our experiment, *B. exitiosa* prevalence increased with ongoing exposure, which was also observed in *O. edulis* exposed to *B. ostreae* infection (Culloty et al., 1999; Montes, 1991) and *C. ariakensis* exposed to *B. exitiosa* (see Audemard et al., 2014). South Australian oyster culture systems are intertidal, which may reduce infection by limiting immersion compared to subtidal culture systems. Intertidal farms, however, are in shallow water with little volume and bi-directional tidal currents which may increase oyster exposure to *B. exitiosa*, rather than deep water with uni-directional currents which would dilute *B. exitiosa* cells and decrease exposure. A detailed examination of *O. angasi* culture systems and their influence on *B. exitiosa* infection is lacking and warrants investigation.

Estimates of prevalence are influenced by diagnostic tests, but diagnoses of *B. exitiosa* in *O. angasi* are well characterised (Buss et al., 2019, Chapter 2) and this understanding facilitates a broader range of analytical approaches to data. The overlap in 95% credible intervals of posterior and prior means (Table 3.2) increases confidence in our estimated prevalences (Table 3.3). The higher posterior predicted means of each test (histology and heart smear) than the estimated prior means (Table 3.2) implies the tests performed better in our study than in Buss et al. (2019, Chapter 2), probably because oysters in this study had higher parasite intensity than most oysters in Buss et al. (2019, Chapter 2).

Bonamia exitiosa intensity increased from day 10, and after day 21 there was evidence of overwhelming parasitaemia. The histological findings in recipient oysters were consistent with the description by Corbeil et al. (2009) of *O. angasi* clinically affected by *B. exitiosa*, supporting that oysters in this study were dying of *B. exitiosa* infection. The *B. exitiosa* intensities we observed were comparable to *B. ostreae* intensities observed on farms showing clinical disease in Europe (Culloty et al., 2004) and were higher than average intensities on farms in South Australia (Buss et al., 2019, Chapter 2). We found no significant difference in *B. exitiosa* intensities between live or dead recipients, but Diggles and Hine (2002) found dead *O. chilensis* had higher *B. exitiosa* intensities than live *O. chilensis.* Diggles and Hine (2002) sampled their animals daily, whereas we sampled every 2–3 days, which may have led to underestimates of the parasite intensity of dead oysters through loss of *B. exitiosa* cells and infected hemocytes as tissue decayed.

The mechanism by which living oysters shed *Bonamia* spp. is undescribed. Host death may facilitate *Bonamia* spp. transmission (Hine & Jones 1994; Hine, 1996) by releasing *Bonamia* spp. cells from decaying oyster tissue, but we observed transmission before the first mortalities, indicating that *B. exitiosa* can be transmitted from living oysters. Stauber (1950) described phagocytic hemocytes throughout the body of oysters. These are particularly common in the epithelium of the gut (Jones, 2011), but they also cross epithelial borders to the exterior of the body

(Cheng, 1996). This process is termed diapedesis (Onstad et al., 2006) and is the most likely mechanism by which hemocytes infected with *Bonamia* spp. are shed from live oysters. The continual loss of hemocytes via diapedesis is normal (Galtsoff, 1964), but increases in the presence of pathogens (Burge et al., 2007; Friedman & Perkins, 1994; Friedman et al., 2005; Heasman et al., 2004). The rate at which hemocytes and therefore *Bonamia* spp. cells are shed through diapedesis and the intrinsic and extrinsic influences on this process, however, remain unknown.

Aspects of the *Bonamia* spp. lifecycle are unknown; the lack of an obligate intermediate host is demonstrated, but it is unclear if facultative intermediate hosts exist or are epidemiologically significant. Lynch, Armitage, Coughlan, Mulcahy and Culloty (2007) detected *B. ostreae* by PCR in pooled zooplankton. This finding needs clarification, however, because PCR cannot differentiate *B. ostreae* cells in the water column or adhering to plankton from those infecting planktonic hosts. If intermediate hosts exist in the *Bonamia* spp. lifecycle they would have an important role in transmission and environmental persistence.

Oysters of all ages are susceptible to *Bonamia* spp. infection (Arzul et al., 2011); larger and older (>20 months) *Ostrea* oysters have higher prevalence, greater mortality (Cáceres-Martínez, Robledo & Figueras, 1995; Culloty & Mulcahy, 1996; Engelsma et al., 2010; Kroeck & Montes, 2005) and higher *Bonamia* spp. intensities (Buss et al., 2019, Chapter 2; Culloty & Mulcahy, 1996), but younger (~12 months) *O. edulis* can still have high *B. ostreae* prevalence (Lallias et al., 2008; Lynch, Armitage, Wylde, Mulcahy, & Culloty, 2005). In *C. ariakensis*, *B. exitiosa* (see Hill et al., 2014) infected younger oysters more rapidly (Carnegie et al., 2008) which also had higher prevalence and displayed greater mortality than older oysters (Bishop, Carnegie, Stokes, Peterson, & Burreson, 2006). Our study confirmed that juvenile *O. angasi* are susceptible to *B. exitiosa* infection and Buss et al. (2019, Chapter 2) has found that adult *O. angasi* are also susceptible. *Ostrea edulis* larvae can acquire *B. ostreae* infection (Arzul et al., 2011) but susceptibility of *O. angasi* larvae to *B. exitiosa* is not established. No studies have compared infection dynamics of a *Bonamia* species between different hosts. It is likely that all life history stages of susceptible oysters can be infected with *Bonamia* spp. but each host-parasite combination is likely to have different infection dynamics. Understanding which life history stages of which hosts can be infected is relevant for surveillance design, translocation assessment and protection of *Bonamia* spp.-free areas.

Our experiment caused rapid infection and progression to clinical disease which provides a basis for testing *O. angasi* for susceptibility to *B. exitiosa*. Selective breeding of family lines for *Bonamia* spp. resistance is a long-term strategy for the management of farmed oysters threatened by *Bonamia* spp. In Ireland, ongoing breeding from *O. edulis* survivors of *B. ostreae* infection

provided oyster stock that could be grown in *B. ostreae*-endemic areas with consistently low prevalence and negligible *B. ostreae*-associated mortality (Lynch, Flannery, Hugh-Jones, Hugh-Jones, & Culloty, 2014). Application of molecular approaches to family line selection (Cao, Fuentes, Comesaña, Casas, & Villalba, 2009; Martín-Gómez, Villalba, & Abollo, 2012) would facilitate faster selection of resistant stock than random selection of survivors through parasite challenge. Investment in a breeding program to select *O. angasi* for resistance to *B. exitiosa* in Australia is justified if substantial industry expansion is anticipated or desired. *Bonamia ostreae* was detected in the southern hemisphere for the first time in New Zealand in 2015 (Lane, Webb, & Duncan, 2016) and *B. ostreae* poses a substantial threat to susceptible oysters in Australia (Animal Health Committee, 2018). The impact of *B. ostreae* in Australia is assessed as severe, but the susceptibility of *O. angasi* to *B. ostreae* and if selectively reared *B. exitiosa*-resistant oysters would also be resistant to *B. ostreae* is unknown. If *B. exitiosa* resistant oysters are also resistant to *B. ostreae*, a breeding program would provide additional insurance against the threat posed by *B. ostreae*.

Cohabitation provides an informative way to mimic natural infection by *Bonamia* spp. and study transmission. We demonstrated rapid infection, with increasing *B. exitiosa* prevalence, intensity, and mortalities in *O. angasi* exposed to an infection source. Given that *B. exitiosa* is widely distributed in southern Australia, this knowledge is important for management and decisions about species diversification for the edible oyster industry in Australia.

Chapter 4 : Infection dynamics of *Bonamia exitiosa* on intertidal *Ostrea angasi* farms.

Figure 4: Oyster farm with a *Pinna bicolor* population below lease, Streaky Bay, South Australia.

Buss, J. J., Wiltshire, K. H., Harris, J. O., Tanner, J. E., & Deveney, M. R. (2020b). Infection dynamics of *Bonamia exitiosa* on intertidal *Ostrea angasi* farms. *Journal of Fish Diseases*. *43*, 359–369, doi: 10.1111/jfd.13134

Abstract

Bonamia spp. cause epizootics in oysters worldwide. In southern Australia, *Bonamia exitiosa* Hine, Cochennac & Berthe, 2001 threatens aquaculture of Native Oysters (*Ostrea angasi* Sowerby, 1871). *Bonamia* spp. infections can display strong seasonality, but seasonal dynamics of *B. exitiosa*-*O. angasi* are unknown. *Ostrea angasi* naïve to *B. exitiosa* infection were stocked onto farms in three growing regions and *B. exitiosa* prevalence and intensity were monitored seasonally for one year. Environmental parameters measured did not correlate with *B. exitiosa* prevalence or infection intensities. Extreme temperatures suggest that *O. angasi* culture systems need development. *Bonamia exitiosa* prevalence increased over time. After three months, *O. angasi* had *B. exitiosa* prevalence of 0.08–0.4 and after one year prevalence was 0.57–0.88. At some sites *Ostrea angasi* had >0.5 *B. exitiosa* prevalence in >6 months but at other sites >9 months passed before prevalence was >0.5. *Bonamia exitiosa* infection intensities were low, with no seasonal pattern but were affected by the interaction of site, season and oyster meat:shell ratio. Understanding infection and initiating a breeding program for resistance would provide benefits for *O. angasi* industry expansion.

4.1 Introduction

Mollusc aquaculture provides a substantial supply of food; global mollusc production in 2016 was 17.1 million tonnes (FAO, 2018). Since the 1970s, diseases caused by the intracellular protozoa *Bonamia* spp. have negatively affected oyster industries in Europe, North America, South America, North Africa, New Zealand and Australia (Arzul et al., 2006; Hill et al., 2014).

A *Bonamia* sp. was identified in Australia in the early 1990s in farmed and wild populations of the Native Oyster (*Ostrea angasi,* Sowerby, 1871), (see Hine & Jones, 1994). Extensive investigations including genome sequencing have identified all southern Australian *Bonamia* isolates as *Bonamia exitiosa* Hine, Cochennac & Berthe, 2001 (see Bradley, 2019; Buss, Wiltshire, Prowse, Harris, & Deveney, 2019, Chapter 2). Handlinger et al. (1999) reported that histological surveys of wild *O. angasi* across South Australia in 1992–93 did not detect *Bonamia*. Surveys of farmed Pacific oysters (*Crassostrea gigas* Thunberg, 1793) in South Australia in 2002, however, reported *Bonamia*-like cells (Diggles, 2003), but no confirmatory diagnosis was made. If these cells were *B. exitiosa*, these data support that *C. gigas* is a host of *Bonamia* spp. as proposed by Lynch et al. (2010). It is therefore likely that *B. exitiosa* was introduced or became widespread in South Australia between 1993 and 2002.

Bonamia spp. transmit directly between hosts (Arzul & Carnegie, 2015). Large farmed oyster populations may have facilitated the proliferation of *B. exitiosa* in South Australia*,* leading to the high prevalences observed by Buss et al. (2019, Chapter 2). Although the origin of South Australian *B. exitiosa* is unknown, it likely arrived via an anthropogenic route. Transmission of *Bonamia ostreae* Pichot, Comps, Tigé, Grizel & Rabouin, 1980 is linked to vessel biofouling (Howard, 1994), translocation of stock (Hudson & Hill, 1991; Peeler, Oidtmann, Midtlyng, Miossec, & Gozlan, 2011) and the seafood trade (Feng et al., 2013). Given that *B. exitiosa* is now established across southern Australia, aquaculture operators and reef restoration proponents need to understand and take the pathogen into account in their planning.

Environmental conditions affect infection dynamics of *Bonamia* spp.-host systems. Salinity is a driver of *Bonamia* spp. infection. High salinity (>20 psu) is associated with higher *B. exitiosa* prevalence and/or infection intensities in *Crassostrea ariakensis* Fujita, 1913 (see Audemard, Carnegie, Bishop, Peterson, & Burreson, 2008a; Audemard et al., 2008b; Audemard, Carnegie, Hill, Peterson, & Burreson, 2014; Bishop, Carnegie, Stokes, Peterson, & Burreson, 2006), higher *B. exitiosa* prevalence and infection intensities in *Ostrea chilensis* Philippi, 1844 (see Hine, Diggles, Parsons, Pringle, & Bull, 2002) and increased survival of *B. ostreae* cells (Arzul et al., 2009). *Bonamia* spp. in different hosts have a range of seasonal patterns of infection. In *B. exitiosa*-*C. ariakensis*, higher *B. exitiosa* associated prevalence and mortality occurs in spring, summer and

early autumn, but *B. exitiosa* was undetectable in *C. ariakensis* at <25°C in late autumn and winter (Carnegie et al., 2008). For *B. exitiosa*-*O. chilensis*, prevalence peaks in autumn and winter (Hine, 1991). In *B. ostreae*-*Ostrea edulis* Linnaeus, 1758 transmission occurs throughout the year, but higher *B. ostreae* prevalence is observed in winter (Arzul et al., 2006; Culloty & Mulcahy, 1996) and spring (Culloty & Mulcahy, 1996; Engelsma et al., 2010).

Field infection dynamics and *B. exitiosa* seasonality are undescribed in *O. angasi*. This study aimed to assess seasonal changes in *B. exitiosa* prevalence and infection intensities in intertidally cultured *O. angasi* over a year. Understanding *B. exitiosa*-*O. angasi* infection dynamics will inform farm management decisions and aid development of the *O. angasi* aquaculture industry.

4.2 Methods

4.2.1 Experimental animals

Common names used are consistent with Australian Fish Names Standard AS 5300-2015 (Standards Australia, 2015).

Fourteen-month-old (7–10 mm) *O. angasi* that had not been used for any experiments were sourced from the South Australian Research and Development Institute (SARDI) Aquatic Sciences Centre (SAASC) Mollusc Hatchery (West Beach, Adelaide, South Australia). Testing by real-time PCR (Corbeil et al., 2006) (n = 150) and histology (n = 150) did not detect *Bonamia* sp. (mean Bayesian estimated prevalence, credible intervals: 0.017, 0.000–0.05, Buss et al., 2019, Chapter 2) and oysters were considered naïve (uninfected and unexposed).

4.2.2 Farm stocking and location

Four oyster farms on the Eyre Peninsula (South Australia) were chosen as experimental sites: Coffin Bay 1, Coffin Bay 2, Cowell and Streaky Bay (Figure 4.1). Approximately 600 juvenile *O. angasi* (total n = 1,780) were stocked in three replicate baskets (15 L, SEAPA, Adelaide, South Australia) and deployed at each of the four sites. At Coffin Bay and Cowell, oysters were deployed in 3 mm mesh size baskets and at Streaky Bay oysters were deployed in 6 mm mesh size baskets. All oysters were chosen from the same cohort and transported to farms within 48 h of leaving the hatchery.

Coffin Bay 1 is in western Coffin Bay. It has a moderate energy environment, is sheltered from prevailing winds, and characterised by a sandy seafloor and high biofouling, comprising mostly barnacles and mussels. Coffin Bay 2 is in the central channel of Coffin Bay and experiences large tidal fluctuations. It is a high energy, deep water site, with a sandy substrate and high biofouling comprising barnacles and mussels. Cowell is in southern Franklin Harbour adjacent to a mangrove (*Avicennia marina* (Forssk.) Vierh.) forest. It is a shallow and low energy environment with a fine sediment substrate and dense *Posidonia australis* Hook. f. beds. The Cowell site has high turbidity and extreme barnacle biofouling with settlement predominantly in summer. Streaky Bay is in southern Streaky Bay and is a shallow, exposed site with high wave and wind energy. This site is characterised by a sandy benthos with *P. australis* and *Amphibolis antarctica* (Labill.) Asch. beds and a *Pinna bicolor* Gmelin, 1791 population. All sites are intertidal; oysters were periodically exposed to air and then submerged under water. In summer during tidal maxima, the substrate at Cowell and Streaky Bay sites was completely exposed. Sites were stocked in the Austral summer in late February 2017 (Table 4.1).

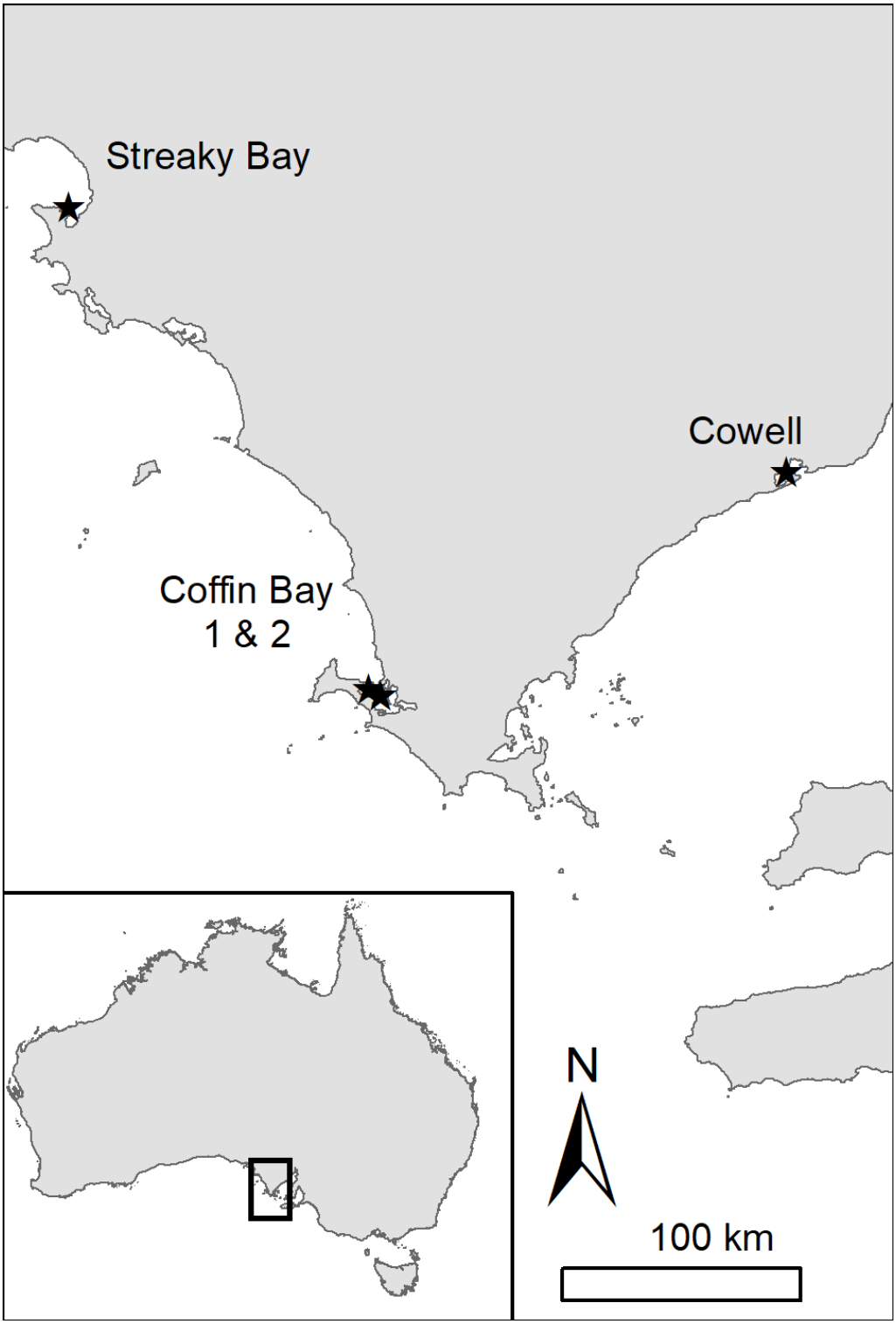

Figure 4.1: ***Ostrea angasi*** **farm stocking locations in the Eyre Peninsula, South Australia. Each star shape represents one farm.**

Table 4.1: Sampling timeline of *Ostrea angasi* from four South Australian farms over four seasons. †

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

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

4.2.3 Experimental design: oyster sampling

To assess the dynamics of *B. exitiosa* in *O. angasi,* the cohort of oysters was monitored seasonally over a year (Table 4.1). Every three months at the end of each Austral season, all baskets were inspected and a sample of 20 oysters per replicate basket (n = 60) were removed from the baskets following a randomisation plan, placed in an ice box, transported ashore and sampled. Oysters were weighed (OHAUS, Scout General (SPX) Portable Balance Scales, Model # SPX223) and shell length measured with a digital caliper (Craftright 150 mm Stainless Steel Digital Vernier Caliper) along the longest shell axis (hinge to top of shell). Oyster meat was removed, the empty shell weighed and the meat to shell ratio calculated (meat weight (g) / shell weight (g) x 100). At the end of the trial, all oysters were removed and survival was estimated.

4.2.4 Diagnostic sampling: heart smears and terminology

Heart smears were used to determine the prevalence and infection intensities of *B. exitiosa* in *O. angasi*. Within 24 h of sampling, heart smears from sample oysters were made on slides, dried, fixed, and stained as per Buss et al. (2019, Chapter 2). Heart smears were viewed under a compound light microscope (Brightfield Olympus BX53) and *B. exitiosa* infection intensities were graded using a semi-quantitative score as per Buss et al. (2019, Chapter 2). Parasitology terminology used for *B. exitiosa* prevalence and infection intensity is consistent with Bush, Lafferty, Lotz, and Shostak (1997).

4.2.5 Experimental design: environmental parameters

Two temperature loggers (HOBO Pendant® Temperature/Light Data Logger 64K-UA-002-64, accuracy ± 0.47°C at 25°C) were used per site, with one placed at the same height as the oyster baskets (the above probe) to measure the temperature oysters were experiencing, and one placed directly below (the below probe) on the benthos to monitor the water temperature. A conductivity logger (Odyssey Conductivity and Temperature Logger, 80 mS/cm, accuracy 3% of reading) was

also used at each site to assess conductivity. Each conductivity logger was placed on the benthos below the oysters. Every three months environmental data were retrieved and collated. Phytoplankton data per season and site were provided by the South Australian Shellfish Quality Assurance Program (SASQAP).

4.2.6 Terms and statistical analyses

Infection intensity data were compared using Quantitative Parasitology 3.0 (Reiczigel, Marozzi, Fábián, & Rózsa, 2019), using Sterne's exact confidence intervals for 95% bootstrap confidence intervals for mean infection intensities (with 5,000 replications). Intensities were considered significantly different when confidence intervals did not overlap.

A Bayesian Latent Class Model (LCM) was built and used to calculate estimated prevalence with credible intervals using JAGS code modified from the prevalence R package (Devleesschauwer et al., 2015). This model used uninformative Beta (1, 1) priors for prevalence estimates. Priors for diagnostic sensitivity (DSe) and diagnostic specificity (DSp) of heart smear were informed by outputs from the analysis of Buss et al. (2019, Chapter 2). These beta priors reflected 95% confidence that heart smear DSe and DSp fall within the credible interval with the mean specified in Table 4.2. Markov Chain Monte Carlo (MCMC) simulations were obtained by running the model in JAGS v. 4.3.0 (Plummer, 2017) using three chains for 10,000 iterations, thinned at a rate of 10, 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 using the Gelman-Rubin convergence statistic, and confirmed by visual inspection of trace, density and autocorrelation plots generated using the MCMCvis package (Youngflesh, 2018). Estimated prevalence values were assessed as being different when credible intervals did not overlap.

A Generalised Linear Model (GLM) was used to assess *B. exitiosa* infection intensities in oysters across site and season. A negative binomial distribution was used for infection intensity analysis due to over dispersion of data relative to a Poisson distribution. Negative binomial GLMs were conducted in R using the MASS package (Venables & Ripley, 2002). *Bonamia exitiosa* infection intensity plots for each site and season were created using the R package ggplot2 (Wickham, 2016).

Parasitology terms used are consistent with Bush, Lafferty, Lotz, and Shostak (1997).

Table 4.2 The beta priors, associated mean and 95% credible intervals for diagnostic sensitivity (DSe) and diagnostic specificity (DSp) for heart smear, used to calculate estimated prevalence, plus the posterior predictions for DSe and DSp of heart smear. †

Test	DSp or DSe	Beta priors	Mean, 95% credible interval	Posterior predication (mean, credible interval)
Heart smear	DSe	(113, 72)	0.61 (0.54–0.68)	0.66 (0.61–0.70)
Heart smear	DSp	(27, 18)	0.60 (0.45–0.73)	0.71 (0.64–0.78)

† Heart smear priors were derived from Buss et al. (2019, Chapter 2).

4.3 Results

4.3.1 Prevalence

Estimated *B. exitiosa* prevalence from heart smears increased over time; estimated prevalence ranged from 0.08–0.40 after three months to 0.57–0.88 after 12 months (Table 4.3). *Ostrea angasi* from Coffin Bay sites 1 and 2 exceeded 0.5 *B. exitiosa* prevalence after six months, whereas those from Cowell and Streaky Bay took nine months for *B. exitiosa* prevalence to exceed 0.5 (Table 4.3). *Ostrea angasi* collected at the end of winter from Coffin Bay 2 had higher estimated *B. exitiosa* prevalence than *O. angasi* from Cowell, but credible intervals for other sites overlapped (Table 4.3). At the end of spring, there were no differences in estimated *B. exitiosa* prevalence in *O. angasi* (Table 4.3).

The posterior predicted means of DSe and DSp for heart smear were higher than the prior means from Buss et al. (2019, Chapter 2) (Table 4.2), but the 95% credible intervals of the posterior predictions overlapped those of the priors.

Table 4.3: Size data (mean ± standard deviation), Bayesian estimated prevalence and mean intensity (calculated through Quantitative Parasitology) of *Bonamia exitiosa* in *Ostrea angasi* from heart smears over four seasons in South Australia. † ‡

		Size data				Bayesian	Quantitative parasitology (bootstrap 95%)
Season	**Site**	**Weight (g) (Mean ± SD)**	**Shell length (mm) (Mean ± SD)**	**Meat:shell ratio (%) (Mean ± SD)**	**n**	**Estimated prevalence (95% credible intervals)**	**Mean heart smear intensity cell count (confidence intervals)**
Autumn	**Coffin Bay 1**	6.02 ± 2.04	39.77 ± 4.78	45.20 ± 8.07	60	0.40 (0.07–0.76)^{ab}	3.65 (2.62–5.04)^{b}
	Coffin Bay 2	6.66 ± 2.13	38.91 ± 4.28	38.63 ± 7.39	60	0.33 (0.03–0.65)^{ab}	4.38 (2.88–7.74)^{ab}
	Cowell	10.17 ± 6.46	45.31 ± 13.52	46.10 ± 11.47	60	0.09 (0.00–0.31)^{b}	3.64 (2.07–5.64)^{ab}
	Streaky Bay	3.05 ± 1.15	29.70 ± 4.12	44.65 ± 6.27	60	0.08 (0.00–0.26)^{b}	4.00 (2.50–7.15)^{ab}
Winter	**Coffin Bay 1**	7.79 ± 4.36	40.74 ± 6.66	41.92 ± 5.84	60	0.53 (0.16–0.88)^{ab}	4.61 (3.56–5.82)^{ab}
	Coffin Bay 2	5.22 ± 2.78	32.76 ± 5.98	37.84 ± 5.90	59	0.88 (0.62–1.00)^{a}	7.02 (5.28–9.67)^{a}
	Cowell	9.62 ± 4.42	44.46 ± 7.52	49.42 ± 10.85	60	0.20 (0.01–0.50)^{b}	3.10 (2.30–4.50)^{b}
	Streaky Bay	3.80 ± 1.44	29.61 ± 4.08	40.48 ± 5.07	60	0.44 (0.10–0.79)^{ab}	3.78 (2.74–5.22)^{b}
Spring	**Coffin Bay 1**	6.36 ± 2.91	38.14 ± 5.51	40.51 ± 5.89	60	0.89 (0.66–1.00)^{a}	4.67 (3.64–6.93)^{ab}
	Coffin Bay 2	6.39 ± 4.17	33.05 ± 6.91	35.86 ± 7.13	59	0.80 (0.49–0.99)^{a}	4.31 (3.50–5.29)^{ab}
	Cowell	9.34 ± 5.44	45.63 ± 8.93	53.06 ± 10.25	60	0.53 (0.16–0.87)^{ab}	3.03 (2.34–3.83)^{b}
	Streaky Bay	5.37 ± 2.68	33.71 ± 7.18	38.67 ± 4.58	59	0.63 (0.26–0.94)^{ab}	4.03 (2.87–7.56)^{ab}
Summer	**Coffin Bay 1**	7.70 ± 4.21	38.07 ± 5.71	35.92 ± 8.38	60	0.88 (0.65–1.00)^{a}	5.51 (4.12–8.36)^{ab}
	Coffin Bay 2	8.44 ± 3.79	37.97 ± 7.57	28.94 ± 6.99	60	0.87 (0.61–1.00)^{a}	5.22 (4.12–7.48)^{ab}
	Cowell	20.69 ± 10.64	53.90 ± 10.56	36.34 ± 7.94	60	0.57 (0.20–0.91)^{ab}	3.37 (2.77–4.20)^{b}
	Streaky Bay	9.24 ± 3.28	42.49 ± 4.98	41.91 ± 4.96	60	0.78 (0.46–0.98)^{ab}	3.36 (2.73–4.11)^{b}

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

‡ SD: standard deviation; n: sample size per site.

4.3.2 Infection intensity

Mean *B. exitiosa* infection intensities did not differ at each site between seasons (Table 4.3). There were no differences between sites within each season except for winter, when *O. angasi* from Cowell and Streaky Bay had lower mean infection intensities than *O. angasi* from Coffin Bay 2 (Table 4.3).

GLM showed a significant three-way interaction between site, season and meat:shell ratio (LRT: $\chi 2$ (9) = 26.12, $p = 1.95e^{-3}$). *Ostrea angasi* from Coffin Bay 2 and Streaky Bay had higher infection intensities associated with higher meat:shell ratio in summer and spring, but in autumn and winter higher infection intensities were associated with lower meat:shell ratio (Figure 4.2). This pattern was not consistent between sites (Figure 4.2).

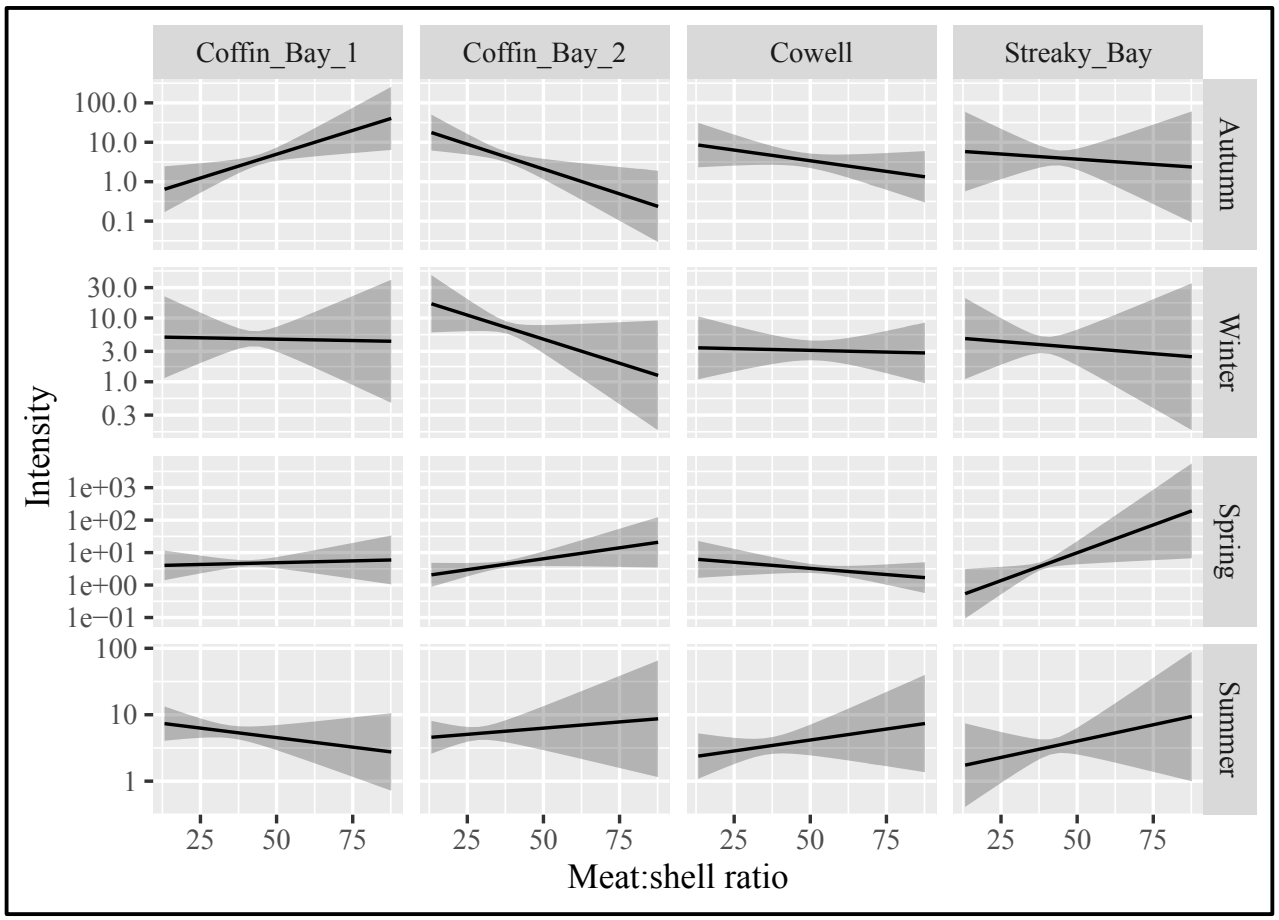

Figure 4.2: *Bonamia exitiosa* intensity in *Ostrea angasi* per site (Coffin Bay sites 1 and 2, Cowell and Streaky Bay), per season (autumn, winter, spring and summer) and the relation to meat:shell ratio.

4.3.3 Survival and size data

The final proportion of survivors at all sites varied between 17.01 and 32.77% (Table 4.4). The lowest proportion of surviving oysters was at Streaky Bay.

After one year, Cowell had larger oysters (20 g mean weight) than other sites (<9 g mean weight (Table 3).

Table 4.4: Count data of *Ostrea angasi* from four South Australia farms after four seasons.

	Initial count at stocking	Final total counts (dead and alive) †	Final counts (live)	Proportion live oysters remaining at end of trial (%)
Coffin Bay 1	1785	685	585	32.77
Coffin Bay 2	1785	399	386	21.62
Cowell	1785	438	414	23.19
Streaky Bay	1775	181	302	17.01

† Final live counts included oysters that were removed for sampling per season.

4.3.4 Environmental parameters

Initial data exploration using a correlation matrix showed infection intensities and prevalence did not correlate with any measured environmental parameter. Cowell experienced the greatest temperature difference: maximum temperature: 48.7ºC, minimum temperature: 5.8ºC. (Table 4.5).

In summer, temperature at Coffin Bay 1 had a maximum of 35ºC, but only December data were available because the probe malfunctioned. At Coffin Bay 2 temperature exceeded 40ºC once. At Cowell, the temperature exceeded 35ºC 19 times and five of these events exceeded 40ºC. Streaky Bay exceeded 35ºC 11 times and five of these events exceeded 40ºC.

Table 4.5: Mean seasonal (Au = autumn, Wi = winter, Sp = spring, Su = summer) water conductivity and temperature data from four South Australian oyster farms (CB1 = Coffin Bay 1, CB2 = Coffin Bay 2, CW = Cowell, SB = Streaky Bay).

	Water conductivity ($mScm^{-1}$) (Mean ± SE) †				**Water temperature: above probe (°C) Mean ± SE)** †				**Water temperature: below probe (°C) (Mean ± SE)** †			
	CB1	**CB2**	**CW**	**SB**	**CB1**	**CB2**	**CW**	**SB**	**CB1**	**CB2**	**CW**	**SB**
Au	54.03±0.16	51.04±0.52	55.65±0.11	54.53±0.16	**18.63±0.03** (13.85–25.71)	**18.75±0.03** (14.90–31.17)	**19.34±0.04** (10.94–31.47)	**19.19±0.04** (7.88–40.07)	**20.77±0.03** (16.71–25.22)	**18.67±0.02** (14.71–23.97)	**19.28±0.03** (11.82–29.75)	**19.34±0.03** (11.14–27.27)
Wi	58.49±0.09	52.44±0.79	55.13±0.20	43.01±0.91	**13.18±0.01** (9.47–18.52)	**13.53±0.07** (7.98–25.03)	**12.82±0.01** (5.76–16.62)	**13.49±0.01** (7.58–22.05)	**13.20±0.01** (9.47–17.00)	**13.35±0.01** (10.75–15.76)	**12.76±0.01** (6.06–15.95)	**13.53±0.01** (9.87–18.71)
Sp	48.81±0.54	48.06±0.65	60.33±0.16	42.48±0.71	**17.68±0.21** (12.21–35.0)	**17.34±0.04** (10.75–41.23)	**17.88±0.04** (9.37–35.01)	**15.74±0.07** (9.77–28.56)	**17.28±0.03** (12.11–24.93)	**17.07±0.03** (11.43–23.20)	**17.98±0.03** (10.26–27.17)	**18.23±0.03** (10.16–34.80)
Su	42.84±1.40	59.14±0.26	63.13±0.15	44.23±2.30	**21.64±0.07** (14.80–32.60)	**22.74±0.02** (15.28–40.07)	**24.21±0.04** (15.66–48.69)	**23.73±0.03** (14.33–48.16)	**22.69±0.02** (17.19–26.68)	**22.64±0.02** (17.38–28.26)	**23.58±0.02** (17.57–32.81)	**23.29±0.02** (14.71–34.90)

† Conductivity probes were positioned at the bottom of the oyster farm post. The temperature probes included one at the top and one at the bottom of the oyster farm post; SE: standard error. Numbers in parentheses list the minimum and maximum temperature for that site and season.

4.4 Discussion

We observed clear patterns of prevalence for *B. exitiosa* in farmed *O. angasi*. *Bonamia exitiosa* was detected in *O. angasi* at all four farm sites within three months of stocking (Table 4.3). *Bonamia exitiosa* prevalence increased in *O. angasi* at all sites over time, with highest prevalence (>0.57) after one year, but *B. exitiosa* infection intensities in *O. angasi* were not different between autumn and summer (Table 4.3). Our findings are consistent with Buss et al. (2019, Chapter 2) who found *B. exitiosa* estimated prevalence of >0.59 and infection intensities of <4.36 in *O. angasi* on South Australian farms. At Cowell and Streaky Bay *B. exitiosa* prevalence in *O. angasi* took nine months to exceed 0.5 whereas Coffin Bay sites exceeded 0.5 infection prevalence within six months of deployment. Sites with slower increases in *B. exitiosa* prevalence may be more suitable for farming *O. angasi*. After one year, Cowell had larger oysters (20 g mean weight, Table 4.3) than other sites (<9 g mean weight, Table 4.3). In warmer water *O. angasi* grows more rapidly (Dix, 1980), and higher temperatures at Cowell (Table 4.5) could explain why oysters stocked in that area were larger than oysters from other sites (Table 4.3). These data indicate that *B. exitiosa–O. angasi* infections are site dependent as described for *B. ostreae* in *O. edulis* populations in Europe (Culloty, Cronin, & Mulcahy, 2004). These data, therefore, can inform decisions about the suitability of sites for oyster aquaculture (Culloty et al., 2004).

Diagnostic tests and their accuracy are well described for *B. exitiosa* in farmed *O. angasi* populations (Buss et al., 2019, Chapter 2). Understanding DSe and DSp of each test means that cost and time effective tests such as heart smear (Diggles, Cochennec-Laureau, & Hine, 2003) can be used and provide more accurate prevalence estimates than for less well characterised tests and provide better interpretation of infection dynamics (Buss et al., 2019, Chapter 2; McDonald & Hodgson, 2018). The posterior predictions of prevalence from heart smears had overlapping credible intervals with the heart smear priors (Table 4.2), indicating that the data in this study are consistent with priors in Buss et al. (2019, Chapter 2). This similarity increases confidence in the estimated prevalences in this study (Table 4.3). Understanding test accuracy improves the certainty of results from field trials and surveillance programs.

Sites with different environments were included in this study, but the environmental parameters we measured did not influence the prevalence or infection intensities of *B. exitiosa*. It is likely that parameters we did not measure, including proximity and density of oysters (Arzul & Carnegie, 2015; Lallias et al., 2008) such as farmed and wild populations of *O. angasi* and possibly *C. gigas* had greater influence than environmental parameters. *Crassostrea gigas* is the predominant oyster farmed in South Australia (Nell, 2001) and a likely host of *Bonamia* spp. (see Diggles, 2003; Lynch et al., 2010). Coffin Bay is the best known and largest oyster farming region in South

Australia with 145 leases (184 ha), followed by Streaky Bay (38 leases, 172 ha) and Cowell (37 leases, 115 ha) (PIRSA, 2017). Populations of farmed *C. gigas* and *O. angasi* in Coffin Bay are larger than in the other regions, which may drive the higher *B. exitiosa* prevalence, but estimates of total farmed oyster population sizes were not available to us. The first record of wild *C. gigas* in South Australia was in 1990 (Hone, 1993) and feral oyster numbers in growing regions can be substantial but are subject to control (EPA, 2005; Wear, Theil, Bryars, Tanner, & de Jong, 2004). *Ostrea angasi* populations are not monitored. The size of wild populations of *O. angasi* and *C. gigas* at each site are, therefore, unknown. Other bivalve species could also be hosts; Coffin Bay has large and growing populations of blue mussel (*Mytilus galloprovincialis* Lamarck, 1819) (unpublished data). Northern hemisphere *M. galloprovincialis* held on a *B. ostreae* affected oyster farm for two years were not infected when assessed by histology (Figueras & Robledo, 1994), but it is unknown if *M. galloprovincialis* can become infected with *B. exitiosa.* Coffin Bay also has a large, commercially fished population of Vongoles (*Katelysia* spp.) (see Dent, Mayfield, & Carroll, 2016). Streaky Bay has large populations of *P. bicolor* on the substrate of the oyster leases and *P. bicolor* shells are a favoured habitat for *O. angasi* settlement (Crawford, 2016). Further investigation of the capacity of other bivalves to act as hosts or reservoirs for *B. exitiosa* could better inform the risk profile for farms.

Contaminants are associated with increased prevalence, mortality and disease in molluscs for a variety of pathogens (Morley, 2010). Mangrove systems capture and retain wastewater-borne contaminants and pollutants (Maiti & Chowdhury, 2013; Tam & Wong, 1995). Environmental contaminants were not measured in this study, but lower prevalence near mangroves in Cowell may be linked to lower environmental contamination at this site.

There were no differences in *B. exitiosa* infection intensities in *O. angasi* over one year; infection intensities of *O. angasi* varied seasonally but with the pattern differing between sites and meat:shell ratio (Figure 4.2). Higher infection intensities in *O. angasi* at Coffin Bay 2 and Streaky Bay were associated with higher meat:shell ratios in spring and summer, but with lower meat:shell ratios in autumn and winter (Figure 4.2). Hine (1991) found that *O. chilensis* in New Zealand had light *B. exitiosa* infections in spring and summer and heavy *B. exitiosa* infections in autumn. Buss et al. (2019, Chapter 2) found higher *B. exitiosa* intensities were associated with lower meat:shell ratios for harvest size 20–22 month-old *O. angasi* in winter. Meat:shell ratio is driven by complex oceanographic and climatic factors that vary seasonally between sites (Grangeré, Ménesguen, Lefebvre, Bacher, & Pouvreau, 2009; Rahman, Henderson, Miller-Ezzy, Li, &, Qin, 2020). Oyster populations undergoing mortality from *Bonamia* spp. infection have poor condition including *O. angasi* (see Corbeil, Handlinger, & Crane, 2009) and *O. edulis* (see Rogan, Culloty, Cross, &

Mulcahy, 1991). Meat:shell ratio may therefore be an indicator of *B. exitiosa* infection severity; poor condition may occur when oysters have advanced infection and better condition may occur when oysters have earlier stages of *B. exitiosa* infection. Data from this study shows oysters from some sites in autumn and winter were prone to lower condition and more advanced stages of *B. exitiosa* infection, but in spring and summer, oysters in better condition were associated with earlier stages of *B. exitiosa* infection. A longer study, or a study with more sites may clarify these differences and reveal if the variability is driven more by season or site.

Hine (1991) found that prevalence and intensity of *B. exitiosa* infection in the gonad increased after spawning in summer. *Ostrea angasi* spawn between mid-spring and early autumn (O'Sullivan, 1980) when they are over two years of age (Crawford, 2016) and brood larvae when they are over 68 mm shell length (O'Sullivan, 1980). Oysters in this study were not old enough to be sexually mature and were not incubating larvae. Seasonal patterns in *B. exitiosa* infections may be more pronounced and consistent in mature *O. angasi* than in the juvenile animals in our experiment.

We found that temperature did not influence *B. exitiosa* prevalence or infection intensities in *O. angasi.* In other *Bonamia* sp.-host systems, the effect of temperature varies; higher water temperature (>20ºC) is associated with increased *B. exitiosa* prevalence and infection intensities in *C. ariakensis* (see Audemard et al., 2014; Carnegie et al., 2008) but *B. ostreae* prevalence increased in *O. edulis* at lower water temperatures (10ºC) (see Cochennec-Laureau & Auffret, 2002). The environment, parasite and host influence infection (Scholthof, 2007) but observational understanding of *B. exitiosa*-*O. angasi* infection dynamics is more useful for management than understanding the effects of individual environmental parameters. Infection dynamics could be better understood by monitoring *B. exitiosa* infection in *O. angasi* in several year classes over multiple years and would aid farm management decisions including timing of stocking, stocking density, site management and husbandry and timing harvest of *O. angasi*.

Survival assessment at the end of the experiment showed that survival after one year was low (17–33%, Table 4.4). *Bonamia exitiosa* infection intensities were lower (3.01–7.02 cells/heart smear) than described for *O. angasi* experiencing clinical *B. exitiosa* disease and mortality (15.40–24.20 cells/heart smear) (Buss, Harris, Tanner, Wiltshire, & Deveney, 2020a, Chapter 3). It is therefore unlikely that oysters were dying from clinical *B. exitiosa* infection in this study. Low *O. angasi* survival (Table 4.4) was not associated with *B. exitiosa* prevalence or infection intensity and was probably caused by suboptimal management of the oysters. Streaky Bay had lower *O. angasi* survival than other sites (Table 4.4), but oysters at this site were cultured in baskets with larger mesh size (6 mm versus 3 mm) and the grower noted loss of oysters within the first season. Farmed *O. angasi* can have high survival (52–92%) when grown subtidally (Mitchell, Crawford, &

Rushton, 2000) and also intertidally (>99%) (Li, Miller-Ezzy, Crawford, Gardner, & Deveney 2019) but optimised approaches to maximising survival in farmed *O. angasi* have not been identified.

In South Australia *O. angasi* are mostly cultivated on intertidal leases in adjustable long-line systems designed for *C. gigas* (see Nell, 2001; Wear et al., 2004). These systems are different to benthic, rack or hanging rope systems used to farm *O. edulis* in Europe (Héral & Deslous-Paoli, 1991). Our temperature data demonstrate that oysters experience extreme conditions in intertidal systems. *Crassostrea gigas* is tolerant of elevated water temperature (Rahman, Henderson, Miller-Ezzy, Li, & Qin, 2019) and can survive acute exposures of up to 34ºC (Holliday, 1995) but 42ºC water temperature for 1 h caused 100% acute mortality (Rajagopal et al., 2005). Information on the thermal tolerance of *O. angasi* is limited; temperatures above 31ºC increase mortality in *O. angasi* larvae (O'Connor, 2015), but optimal and lethal temperatures for other *O. angasi* life stages have not been assessed. *Ostrea angasi* is a subtidal species (Edgar, 2008) and is likely to have a lower upper temperature tolerance than intertidal adapted *C. gigas*. Intertidal oyster farms are more prone to impacts of climate change and a breeding program could select traits that facilitate better production in challenging climates (Leith & Haward, 2010). *Ostrea angasi* reefs are being restored across southern Australia (Gillies, Crawford, & Hancock, 2017) and are also at risk from *B. exitiosa* infection. *Bonamia exitiosa* transmission between oyster farms and restored reefs could create mutual disease risk. To date, however, Australian sites for reef restoration have been approved based on oceanographic modelling and ecosystem based development principles to avoid increasing pathogen and biosecurity risks. *Ostrea angasi* farming and reef restoration could also co-expand if careful management is applied. Susceptible-infected-parasite models (Bidegain et al., 2017) simulating *Perkinsus marinus* (Mackin, Owen, & Collier) Levine, 1978 infection dynamics between wild and farmed *Crassostrea virginica* Gmelin, 1791 show that farmed *C. virginica* could act as a pathogen sink by removing parasites if the farmed population was harvested before onset of clinical disease and shedding of parasites (Ben-Horin et al., 2019). Timing harvests may therefore provide an alternate management strategy and facilitate expansion of both *O. angasi* aquaculture and reef restoration, but an understanding of parasite uptake and shedding prior to onset of clinical signs and optimal harvest times would need to be established to develop practically implementable farm management.

Aquaculture and reef restoration could benefit from a program to breed for resistance against *B. exitiosa*. In Rossmore, Ireland, an *O. edulis* breeding program produces *B. ostreae* resistant *O. edulis*. *Bonamia ostreae* prevalence decreased from 90% to <15% with negligible mortality in selected stock. This program, however, was based on mass selection and breeding from survivors

and took 30 years to achieve substantial *B. ostreae* resistance (Lynch, Flannery, Hugh-Jones, Hugh-Jones, & Culloty, 2014). If the Australian oyster industry chose to expand *O. angasi* production, a breeding program incorporating an infection model to test susceptibility and molecular methods to identify markers of resistance could decrease time to achieving resistant stock. Such a program would be expensive, and cost-benefits would need to be examined to justify the expense.

This is the first analysis of *B. exitiosa* infection dynamics in *O. angasi*. *Bonamia exitiosa* occurred at all our study sites and although *O. angasi* from some sites had slower increases in *B. exitiosa* prevalence and may be more suited to *O. angasi* cultivation, *O. angasi* at all sites had >0.5 prevalence after one year. Intensification of *O. angasi* farming at Cowell and Streaky Bay may, however, change infection dynamics. Reef restoration projects also need to continue to be well planned to ensure that they are not developed where they can negatively interact with oyster farms. Pairing site-specific information with a program to breed for resistance against *B. exitiosa* would aid expansion of *O. angasi* aquaculture.

Chapter 5 : Decontamination of *Bonamia exitiosa*.

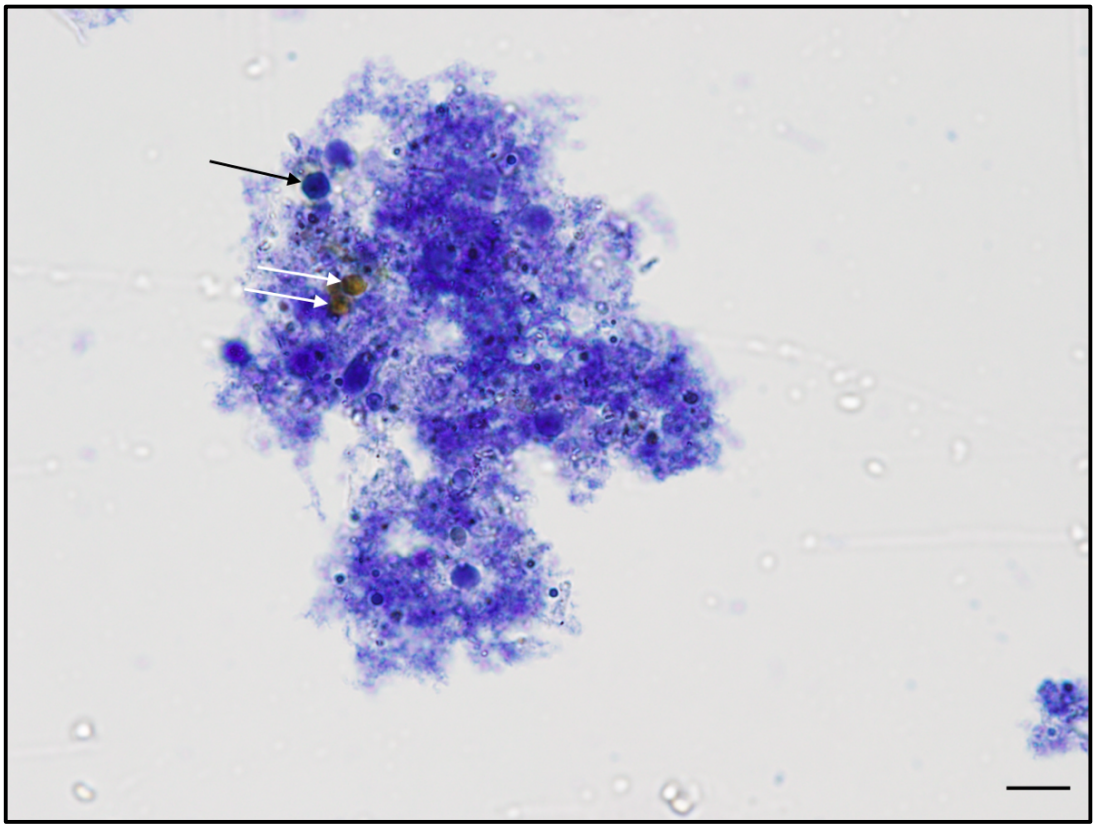

Figure 5: Purified *Bonamia exitiosa* cells. White arrows highlight live cells and the black arrow highlights a dead cell. Scale bar = 10 µm. Trypan Blue staining.

Buss, J. J., Wiltshire, K. H., Harris, J. O., & Deveney, M. R. (under review, submitted 27 October 2019). Decontamination of *Bonamia exitiosa*. *Aquaculture*.

5.1 Abstract

Mollusc aquaculture worldwide has suffered significant losses from disease caused by haplosporidian parasites. *Bonamia exitiosa* Hine, Cochennac & Berthe, 2001 has caused significant epizootics in oyster populations in New Zealand, America and Australia and threatens oyster industry expansion. Decontamination of equipment is an important management tool to limit spread of infection. There are no decontaminants with a regulatory authority for controlling *B. exitiosa* and no efficacy data are available. We assessed the efficacy of three disinfectants permitted by the Australian Pesticides and Veterinary Medicines Authority for decontaminating ostreid herpes virus-1 (OsHV-1) and other oyster pathogens against *B. exitiosa*. Purified *B. exitiosa* cells from infected *Ostrea angasi* Sowerby, 1871 were exposed to a quaternary ammonium compound (QAC), an iodine or chlorine based disinfectant for 1 min, 5 min or 10 min. After disinfectant exposure cell viability was determined using trypan blue staining and light microscopy. 40,000 ppm chlorine for 10 min and 2,000 ppm iodine for 1 min provided 100% efficacy against *B. exitiosa*. QAC did not effectively decontaminate *B. exitiosa*, but QAC can be used for cleaning before decontamination. Understanding how to decontaminate *B. exitiosa* will aid development of management strategies for *B. exitiosa* in industry and laboratories.

5.2 Introduction

Molluscs are important global aquaculture species with 17.1 million tonnes produced in 2016 (FAO, 2018). Semi-open culture systems (Department of Agriculture, 2015a), used for oyster farming increase oyster vulnerability to many aquatic pathogens (Pernet, Lupo, Bacher, & Whittington, 2016). Mollusc culture worldwide has been negatively affected by protozoan parasites including *Haplosporidium nelsoni* Haskin, Stauber & Mackin, 1966 (MSX), *Perkinsus marinus* (Mackin, Owen, & Collier) Levine, 1978 (Dermo) and *Bonamia* spp. which cause substantial losses in mollusc industries worldwide (Arzul & Carnegie, 2015).

Transport of live hosts poses the highest risk for disease introduction (McKindsey et al., 2007), but mechanical transport of pathogens on equipment, personnel or vehicles is an important route of pathogen introduction to new areas, particularly if environmental conditions in transport are suitable for pathogen survival (Peeler & Thrush, 2009). Implementing biosecurity for semi-open oyster farms is difficult and intervention is typically limited to general containment and control measures including zoning, stock movement controls, farming to minimise disease, and decontamination of equipment and fomites (Department of Agriculture, 2015b). Decontamination is the process that involves cleaning and destruction infective agents (DAFF, 2008) and is an essential part of biosecurity activities to limit spread of pathogens associated with equipment and fomites. Disinfectants are products that inactivate pathogens (DAFF, 2008). Disinfectants are not treatments for use on livestock but information on disinfectant efficacy is important to ensure that decontamination facilitates safe movement of personnel and equipment between zones (OIE, 2019a). Disinfecting agents suitable for aquatic animal industries include oxidising agents, pH modifiers (alkalis and acids), aldehydes, biguanides, quaternary ammonium compounds (QACs), ultraviolet light, ozone, heat, drying and high temperatures (DAFF, 2008). There is, however, a need to understand species-specific decontamination procedures for agents that cause infectious diseases.

Numerous disinfectants have been trialled on parasitic marine protozoans (Table 5.1) but disinfectants for *Bonamia* spp. are not well documented; *Bonamia ostreae* Pichot, Comps, Tigé, Grizel & Rabouin, 1980, was effectively decontaminated by ozone at 3.5 mg/L for 60 min or a 1 ppt peracetic acid and hydrogen peroxide solution (Bactipal®, SEPPIC, France) for 30 min (Sindermann, 1984), but Bactipal® is not registered in Australia and there was no information on disinfectants for *Bonamia exitiosa* Hine, Cochennac & Berthe, 2001.

Bonamia ostreae and *B. exitiosa* are listed as notifiable diseases by the World Organisation for Animal Health (OIE) (OIE, 2019b, c). Unlike other haplosporidians, *B. exitiosa* and *B. ostreae* do not have spore stages (Carnegie et al., 2006), which is likely to affect dose and duration of

treatment required for decontamination. *Bonamia ostreae* can survive in seawater for 48 h (Arzul et al., 2009) and makes transport of equipment or personnel exposed to *Bonamia*-infected seawater a risk for *Bonamia* translocation to new areas. *Bonamia exitiosa* has a global distribution in oyster populations including Australia (Bradley, 2019; Buss, Wiltshire, Prowse, Harris, & Deveney, 2019, Chapter 2), New Zealand (Hine, Cochennec-Laureau, & Berthe, 2001), Europe (Abollo et al., 2008), North America, South America and North Africa (Hill et al., 2014) and substantial *B. exitiosa* epizootics have occurred in New Zealand (Cranfield, Dunn, Doonan, & Michael, 2005), America (Audemard, Carnegie, Hill, Peterson, & Burreson, 2014) and Australia (Handlinger et al., 1999). Decontamination data for *B. exitiosa* would therefore aid multiple oyster industries worldwide.

Disinfectants suitable for use on oyster farms should be readily accessible, available in sufficient quantities, safe and products for which regulatory authority for use can be obtained with minimal difficulty. Two current minor use permits issued by the Australian Pesticides and Veterinary Medicines Authority (APVMA) are for decontamination of oyster equipment. PER 14029 authorises chlorine for general decontamination. PER 82160 authorises iodine, sodium hydroxide, QAC or other products containing potassium peroxomonosulphate triple salt, sodium dodecyl benzene sulphonate and sodium chloride for decontamination of ostreid herpes virus-1 microvariant (OsHV-1).

Chlorine, iodine, and QACs were chosen for assessment as disinfectants against *B. exitiosa* because they are already have regulatory authority for use in Australia, are accessible and have low workplace safety risk profiles. QACs have low toxicity (Wild, 2017), dissolve well in water (Rajkowska et al., 2016), are non-corrosive, have low odour and are non-irritant. The efficacy of QACs, however, can be decreased by hard water (DAFF, 2008; Wild, 2017) or combination with anionic agents such as detergents or soaps (DAFF, 2008; Wild, 2017). Chlorine and iodine have higher workplace safety risk as they can cause irritation (Rutala & Weber, 2017; Wild, 2017) or serious injury if consumed (Barnes & Greive, 2013). Iodine exposure causes irritation and excessive staining (McDonnell & Russell, 1999), but neutralisation with sodium thiosulfate limits irritation (Kondo et al., 2001) and removes stains (Gignac et al., 2016).

Table 5.1: Disinfectant treatments that provided successful decontamination against protozoans. n/a: Not applicable.

Protozoan	Protozoan life stage	Disinfectant	Treatment	Result	Reference
Perkinsus marinus	n/a	Chlorine	300 ppm chlorine, 30 min	100% efficacy	(Bushek, Holley, & Kelly, 1997)
Perkinsus atlanticus	1) Free zoospores 2) Free prezoosporangia 3) Prezoosporangia in gill tissue	Chlorine	1) 50 ppm chlorine, 1 h 2) 200 ppm chlorine, 1h 3) 3,000 ppm chlorine, 1 h	100% efficacy	(Casas, Villalba, & Reece, 2002)
Perkinsus sp.	Free prezoosporangia	Chlorine	6 ppm chlorine, 30 min	100% efficacy	(Goggin, Sewell, & Lester, 1990)
P. marinus	n/a	Organic N-halamines: 1) DC† 2) MC†	1) 14.9 ppm DC, 8 h 2) 24.9 ppm MC, 12 h	100% efficacy	(Delaney, Brady, Worley, & Huels, 2003)
P. marinus	Hypnospore	Organic compounds: 1) Avatec 2) Robenz 3) Biocox 4) Monteban 5) Coban 6) Monensin 7) Quinine sulfate 8) Nitrofurazone 9) Acriflavine 10) Malachite green 11) Mertect 340F 12) Captan 13) Benomyl 14) Cycloheximide	Five days of each chemical in culture: 1) 100 ppm Avatec 2) 100 ppm Robenz 3) 100 ppm Biocox 4) 50 ppm Monteban 5) 200 ppm Coban 6) 10 ppm Monensin 7) 50 ppm Quinine sulfate 8) 100 ppm Nitrofurazone 9) 10 ppm Acriflavine 10) 10 ppm Malachite green 11) 110 ppm Mertect 340F 12) 100 ppm Captan 13) 100 ppm Benomyl 14) 50 ppm Cycloheximide	100% efficacy	(Krantz, 1994)
1) *P. marinus* 2) *Haplosporidium nelsoni*	n/a	Ultra violet radiation (30,000 µW/s/cm), coupled with particle filtration (1 µM)	Continuous filtration/UV disinfection of water supplied to *Crassostrea virginica* larvae	100% efficacy for *P. marinus* and *H. nelsoni*	(Ford, Xu, & Debrosse, 2001)
Marteilia sydneyi	Spores	Chlorine	200 ppm chlorine, 4 h	100% efficacy	(Wesche, Adlard, & Lester, 1999)

Different biocidal components are responsible for disinfectant ability; elemental iodine (I_2) and hypoiodous acid (HOI) are responsible for iodine's biocidal characteristics and are available below pH 9 (Black, Kinman, Keirn, Smith, & Harlan, 1970; NRC, 1980). When sodium hypochlorite (NaOCl) is added to water, both hypochlorous acid (HOCl) and hypochlorite ions (OCl^-) are formed (Fair, Morris, Chang, Weil, & Burden, 1948). HOCl has better biocidal properties than OCl^- (Brazis, Leslie, Kabler, & Woodward, 1958; Death & Coates, 1979; Fukuzaki, 2006; Robeck, 1981) and HOCl formation is favoured between pH 4–7 (Black, Lackey, & Lackey, 1959; Wang et al., 2007). Hypochlorite disinfectants, like iodine, enter cells and oxidise cell components leading to cell lysis (Fukuzaki, 2006; McDonnell & Russel, 1999). QAC decontamination capacity is low and selective (QACs can decontaminate gram-positive bacteria, some fungi, but not all viruses and not spores), but is preferred for sanitation purposes, particularly because QACs are sufficiently non-corrosive and safe to use on human skin (DAFF, 2008). This is important for decreasing biosecurity risk associated with personnel movement.

This study aimed to assess the efficacy of iodine, chlorine and QAC as disinfectants against *B. exitiosa* and to define dose and exposure criteria for 100% efficacy. Using products currently permitted by APVMA makes obtaining regulatory authorities for these products in Australia simpler, and is likely to provide more rapid access to disinfectants for farmers. Australian MUPs reflect availability of data that can also be used to obtain regulatory authorities for products in other countries. Effective decontamination methods for *B. exitiosa* is important for management and biosecurity on oyster farms and laboratories globally.

5.3 Methods

5.3.1 Source of experimental animals and live feeds

Ostrea angasi Sowerby, 1871 were sourced from a farm in Grassy Point, Victoria which Bradley (2019) identified as a site infected with *B. exitiosa* using genomic sequencing, and held at the South Australian Aquatic Biosecurity Centre (SAABC) for 21 months. Oysters were maintained in 500 L tanks with continuous aeration and fed 15 L of concentrated mixed live algae culture (*Chaetoceros muelleri*, Lemmermann*,* 1898*, Skeletonema costatum,* Greville, 1873 and *Pavlova lutheri*, Droop, 1975) per tank every two to three days.

5.3.2 Disinfectant products and treatments

Exposures in this study were based on minor use permits (MUPs) PER14029 and PER82160 authorised by the Australian Pesticides and Veterinary Medicines Authority (APVMA).

A ranging study for NaOCl (8–12.5% available chlorine, Chem-Supply) exposed *B. exitiosa* cells to a 0 ppm seawater control and 1 ppm, 10 ppm, 100 ppm, 1,000 ppm, and 10,000 ppm free chlorine for 1 min, 5 min and 10 min. Cell viability in the 10,000 ppm treatment could not be assessed in the ranging study because 10,000 ppm free chlorine bleached the visualisation dye. In a separate chlorine assessment, *B. exitiosa* cells were exposed to 10,000 ppm free chlorine (NaOCl, 8–12.5% available chlorine, Chem-Supply) as well as higher chlorine concentrations (20,000 ppm and 40,000 ppm) for 1, 5 and 10 min.

QAC (Detsan detergent sanitiser, Chemetall) was assessed by exposing *B. exitiosa* cells to a 0 ppm seawater control and 250 ppm, 1,000 ppm and 2,000 ppm available free quaternary ammonium for 1, 5 and 10 min.

An iodine based disinfectant (Agridyne, Tasman Chemicals) was assessed by exposing *B. exitiosa* cells to a seawater control and 10 ppm, 100 ppm, 1,000 ppm, 2,000 ppm, 4,000 ppm and 8,000 ppm free iodine for 1, 5 and 10 min.

All trials included three replicates per time-concentration combination. At the completion of each chlorine or iodine treatment sodium thiosulfate (Chem-supply) was used to neutralise free chlorine or iodine. Without neutralisation, free chlorine and iodine treatments would affect the trypan blue and prevent differentiation of dead and living *B. exitiosa* cells. Where sodium thiosulfate was used, a sodium thiosulfate control was included to assess the effect of sodium thiosulfate on *B. exitiosa* viability. Sodium thiosulfate doses were based on OIE (2009).

5.3.3 Purification

Ten oysters were pre-screened, using heart smears following the method of Diggles, Cochennec-Laureau, and Hine (2003) as used by Buss et al. (2019, Chapter 2). All oysters were positive for *B. exitiosa* with light intensity (2–10 cells/smear). Tissue from each oyster excluding the adductor muscle was maintained in a 50 mL falcon tube at 4°C. Tissue from seven oysters with highest heart smear intensities were homogenised (TissueRuptor®, Qiagen) and used for *B. exitiosa* cell purification following the Diggles and Hine (2002) modification of the procedure described by Mialhe, Bachère, Chagot and Grizel (1988). Purified *B. exitiosa* cells were re-suspended in autoclaved, filtered (0.2 μm) seawater (pH 7, salinity 40 psu), aliquoted into separate 1.5 mL tubes and maintained at 4°C for 12 h prior to use for each trial. Temperature and salinity conditions for *B. exitiosa* cells were based on conditions in South Australian oyster farming regions (Kämpf & Ellis, 2015), which are consistent with conditions that favour high *Bonamia* cell survival (Arzul et al., 2009).

5.3.4 Experimental protocol

On each trial day, QAC, chlorine and iodine stock solutions were made at double each specified concentration because the solution would undergo 1:2 dilution when the *B. exitiosa* cells were added. Treatment containers were wrapped in aluminium foil to limit the degrading effects of light. Total free chlorine and iodine were measured using test strips (for chlorine: WaterWorks™ Ionide CAT: 480024 and 480022; for iodine: WaterWorks™ Ionide CAT: 480064) to ensure the accuracy of concentrations of treatments and to ensure that the sodium thiosulfate effectively neutralised the free iodine/chlorine. For each aliquot of purified cells an equal volume of disinfectant was added and the sample was vortexed periodically throughout exposure to each time treatment. At the conclusion of each exposure, 0.4% liquid trypan blue stain (Sigma Aldrich, CAT: T8154) was added to each aliquot matching the aliquot volume. Each aliquot was lightly vortexed and 10 μL of cells with trypan blue was loaded onto a hemocytometer to count viable and dead cells under a light microscope (Brightfield Olympus BX53) at 400 x. *Bonamia exitiosa* cells were considered viable if they did not take up the dye, whereas those that did take up the trypan blue stain were considered inviable (Diggles & Hine, 2002). To account for the possibility of *B. exitiosa* cells dying prior to disinfectant exposure, *B. exitiosa* cells were exposed to the highest disinfectant dose first, and the seawater control last to avoid the control having higher cell viability. For each treatment replicate >100 cells (total live and/or dead cells) were examined within 3 min. Mean cell viability (%) was calculated = mean viable cells / (mean viable cells + mean dead cells) * 100. Disinfectant efficacy per treatment was calculated as percentage reduction of mean cell viability

using the following formula, modified from Stone, Sutherland, Sommerville, Richards and Endris (2000): Treatment efficacy = 100 – (100 * (mean cell viability of treatment / mean cell viability for the seawater control). All experiments were conducted at 20ºC air temperature.

5.3.5 Statistical analyses

Bayesian logistic regression generalised linear models (GLMs) are an appropriate method for analysis for binomial data (Zuur, Ieno, & Hilbe, 2013) and were used to compare patterns in cell viability for time and concentrations treatments for chlorine, iodine and QAC disinfectants. For each analysis, exposure time and disinfectant treatment were included as factors. Diffuse normal priors, with mean of zero and precision of 0.0001, were used for parameter estimates in models for the chlorine ranging experiment and QAC trial. Some time-treatment combinations for the assessment of higher chlorine concentrations and the iodine assessment had no viable cells, leading to complete separation in the data and hence models using diffuse normal priors did not converge. For these models, therefore, minimally informative prior were used, specifically, scaled *t* priors, centred on zero, with scale of 25 for the intercept and 10 for covariate coefficients, and 7 degrees of freedom, following Gelman, Jakulin, Pittau, and Su (2008) and Ghosh, Li, and Mitra (2018). Models with and without an interaction term were compared by Deviance Information Criterion (DIC) value to determine whether interaction terms were important. Treatment and time effects were assessed by determining whether 95% credible intervals of posterior predictions overlapped. All analyses were conducted in R (R Core Team, 2017). 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 50,000 iterations for burn-in. Convergence was assessed using the Gelman-Rubin convergence statistic, and confirmed by visual inspection of trace, density and autocorrelation plots generated using the MCMCvis package (Youngflesh, 2018). JAGS was run using the R2jags package (Su & Yajima, 2015). Plots to assess cell viability for concentration and time treatments were created using the R function ggplot2 (Wickham, 2016). Plots were generated using predictions from the model including the time–concentration interaction in each case.

We defined disinfectants that had clear credible interval separation from the control and 100% efficacy as effective treatments against *B. exitiosa*.

5.4 Results

5.4.1 Chlorine ranging study

In the chlorine ranging study the best model fit (lowest DIC) showed an interaction between time and concentration (Table 5.2). The effect of chlorine on *B. exitiosa* cell viability varied with time and concentration; chlorine doses of 100 ppm and 1,000 ppm had overlapping credible intervals and lower *B. exitiosa* cell viability than chlorine treatments <10 ppm (Figure 5.1). There was no clear pattern in *B. exitiosa* viability over differing exposures (Figure 5.1). *Bonamia exitiosa* viability in the seawater control decreased over time, but was higher than all chlorine treatments except 1 ppm chlorine for 10 min (Figure 5.1). Maximum efficacy against *B. exitiosa* was 70.72% after 10 min exposure to 1,000 ppm chlorine (Table 5.3).

Table 5.2: Deviance Information Criterion (DIC) for different Bayesian models incorporating decontaminant concentrations and times (1 min, 5 min and 10 min).

Trials[§]	Concentration (F) * Time (F) [† ‡]	Concentration (F) + Time (F) [† ‡]
Chlorine (1)	**316**	320
Chlorine (2)	214	**202**
QAC	205	**192**
Iodine	266	**260**

†Where (F) signifies Factor; * signifies an interaction; + signifies no interaction.

‡ Lower DIC signifies better model fit. Best model fit is signified in bold.

§ Chlorine (1) trial included: Sea water control, 1 ppm, 10 ppm, 10 ppm and 1,000 ppm free chlorine; Chlorine (2) trial included: Sea water control, sodium thiosulfate control, 10,000 ppm, 20,000 ppm, and 40,000 ppm free chlorine; Quaternary Ammonium Compound (QAC) trial included: Sea water control, 250 ppm, 1,000 ppm, and 2,000 ppm free quaternary ammonium; Iodine trial included: Sea water control, sodium thiosulfate control, 10 ppm, 100 ppm, 1,000 ppm, 2,000 ppm, 4,000 ppm and 8,000 ppm free iodine.

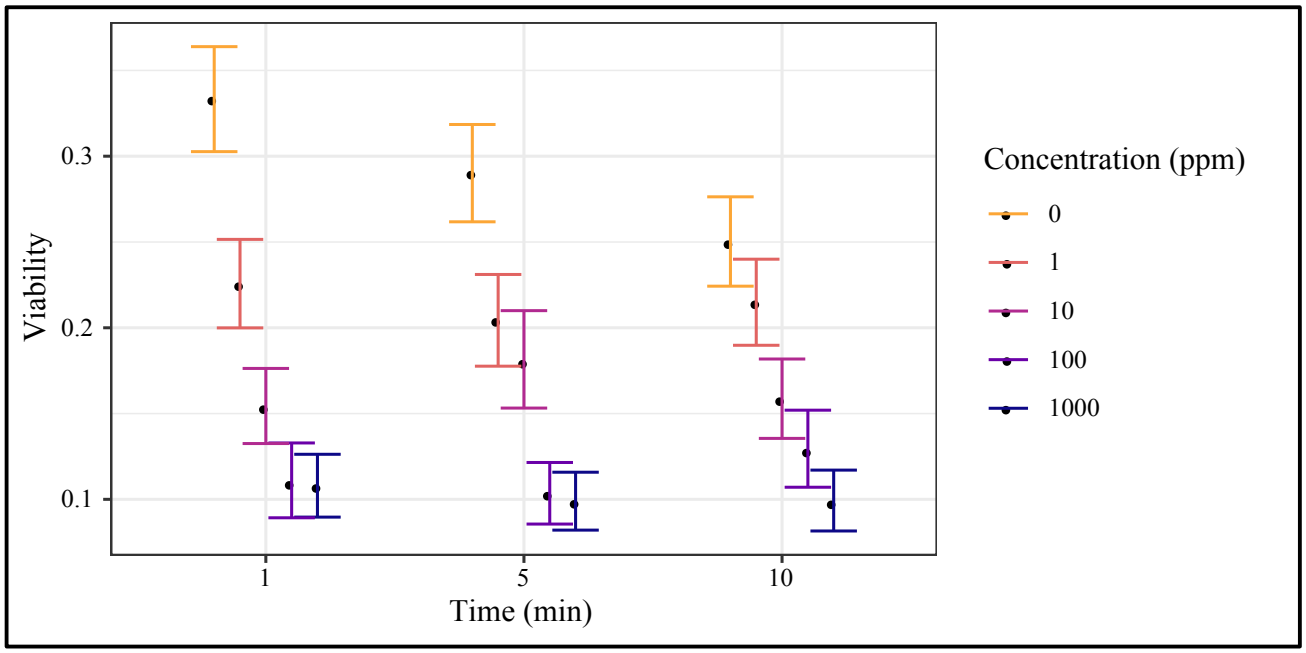

Figure 5.1: *Bonamia exitiosa* cell viability after exposure to seawater control (0 ppm) or free chlorine (1 ppm, 10 ppm, 100 ppm and 1,000 ppm) for 1 min, 5 min and 10 min. Bayesian estimated mean, 95% credible intervals.

Table 5.3: Mean *Bonamia exitiosa* cell viability (%) and treatment efficacy (%) per time (1 min, 5 min and 10 min) and concentration treatment of free chlorine (1 ppm, 10 ppm, 100 ppm, and 1,000 ppm) and the seawater (0 ppm) control for the chlorine ranging study.

Time (min)	Chlorine concentration (ppm)	Mean cell viability (%) †	Treatment efficacy (%)
1	**0**	33.72 ± 2.00	0.00
	1	22.21 ± 1.08	34.12
	10	15.66 ± 0.97	53.55
	100	10.98 ± 0.43	67.45
	1,000	10.75 ± 0.24	68.13
5	**0**	30.50 ± 2.78	9.54
	1	20.26 ± 0.62	39.91
	10	18.10 ± 0.16	46.33
	100	10.25 ± 0.31	69.59
	1,000	10.01 ± 0.80	70.31
10	**0**	24.84 ± 2.44	26.33
	1	22.27 ± 2.32	33.97
	10	15.97 ± 1.19	52.63
	100	12.86 ± 0.49	61.85
	1,000	9.87 ± 0.31	70.72

† Cell viability values were mean ± SE; n = 3.

5.4.2 Chlorine assessment

In the best model fit for the chlorine assessment, there was no interaction between time and concentration (Table 5.2). There were clear differences in viability between different chlorine doses, but for the same chlorine dose credible intervals overlapped for different time exposures (Figure 5.2). There were no differences in viability between seawater and sodium thiosulfate controls, which had higher cell viability than any chlorine treatment (Figure 5.2). The cell viability in the 10,000 and 20,000 ppm chlorine treatments did not differ from each other and had lower viability than both controls (Figure 5.2). The 40,000 ppm chlorine dose was 100% effective at 10 min exposure and all 40,000 ppm chlorine treatments had lower *B. exitiosa* viability than all controls and all other chlorine treatments (Figure 5.2, Table 5.4).

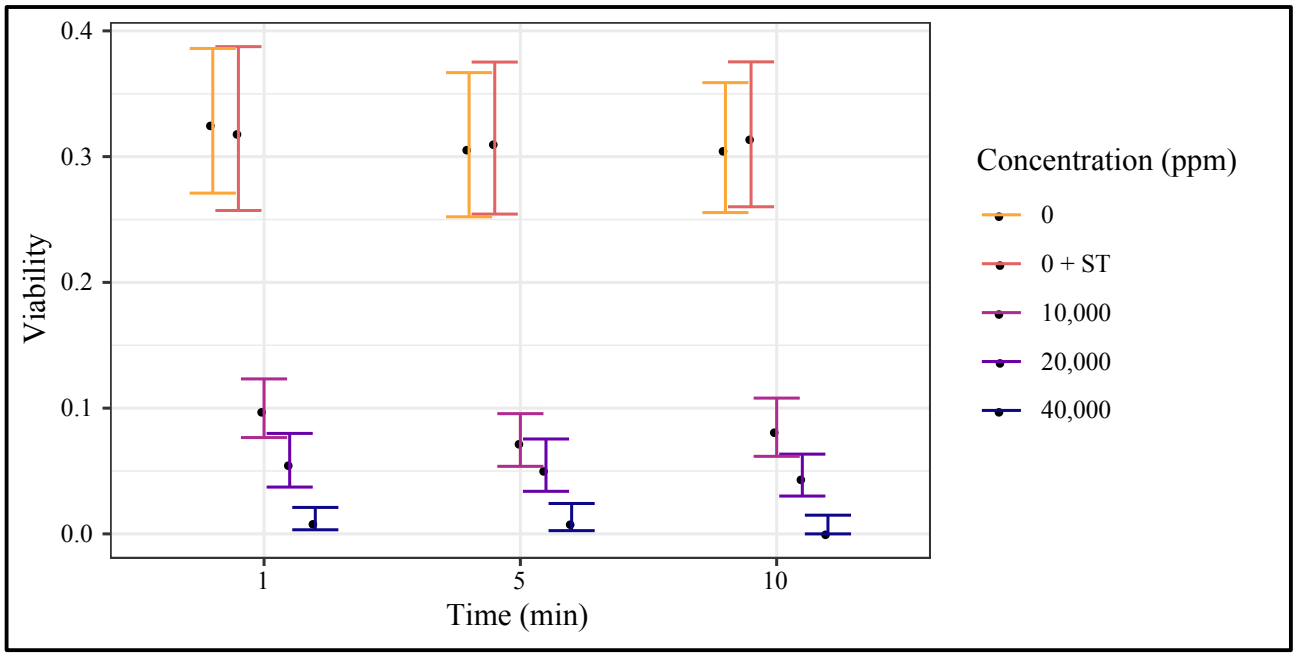

Figure 5.2: *Bonamia exitiosa* cell viability after exposure to seawater control (0 ppm), sodium thiosulfate control (0 ppm + ST) or free chlorine (10,000 ppm, 20,000 ppm and 40,000 ppm) for 1 min, 5 min and 10 min. Bayesian estimated mean, 95% credible intervals.

Table 5.4: Mean *Bonamia exitiosa* cell viability (%) and treatment efficacy (%) per time (1 min, 5 min and 10 min) and concentration treatment of free chlorine (10,000 ppm, 20,000 ppm and 40,000 ppm), the sodium thiosulfate (ST) or seawater (0 ppm) controls for the chlorine assessment.

Time (min)	Chlorine concentration (ppm)	Mean cell viability (%) †	Treatment efficacy (%)
1	**0**	32.88 ± 1.82	0.00
	0 (ST)	31.79 ± 1.00	3.30
	10,000	9.95 ± 1.19	69.73
	20,000	5.60 ± 0.58	82.95
	40,000	0.97 ± 0.22	97.05
5	**0**	30.70 ± 0.38	6.62
	0 (ST)	31.26 ± 0.97	4.93
	10,000	7.36 ± 0.17	77.61
	20,000	5.47 ± 1.00	83.37
	40,000	0.80 ± 0.40	97.57
10	**0**	30.94 ± 2.11	5.91
	0 (ST)	31.46 ± 0.94	4.33
	10,000	8.14 ± 0.97	75.24
	20,000	4.55 ± 1.31	86.17
	40,000	0.00 ± 0.00	100.00

† Cell viability values were mean ± SE; n = 3.

5.4.3 QAC assessment

In the best model fit for the QAC assessment there was no interaction between time and concentration (Table 5.2). Credible intervals for viability for the seawater control and all QAC treatments overlapped (Figure 5.3). Increasing QAC concentration did not decrease *B. exitiosa* cell viability (Figure 5.3, Table 5.5). Efficacy for all QAC concentrations was <27% (Table 5.5).

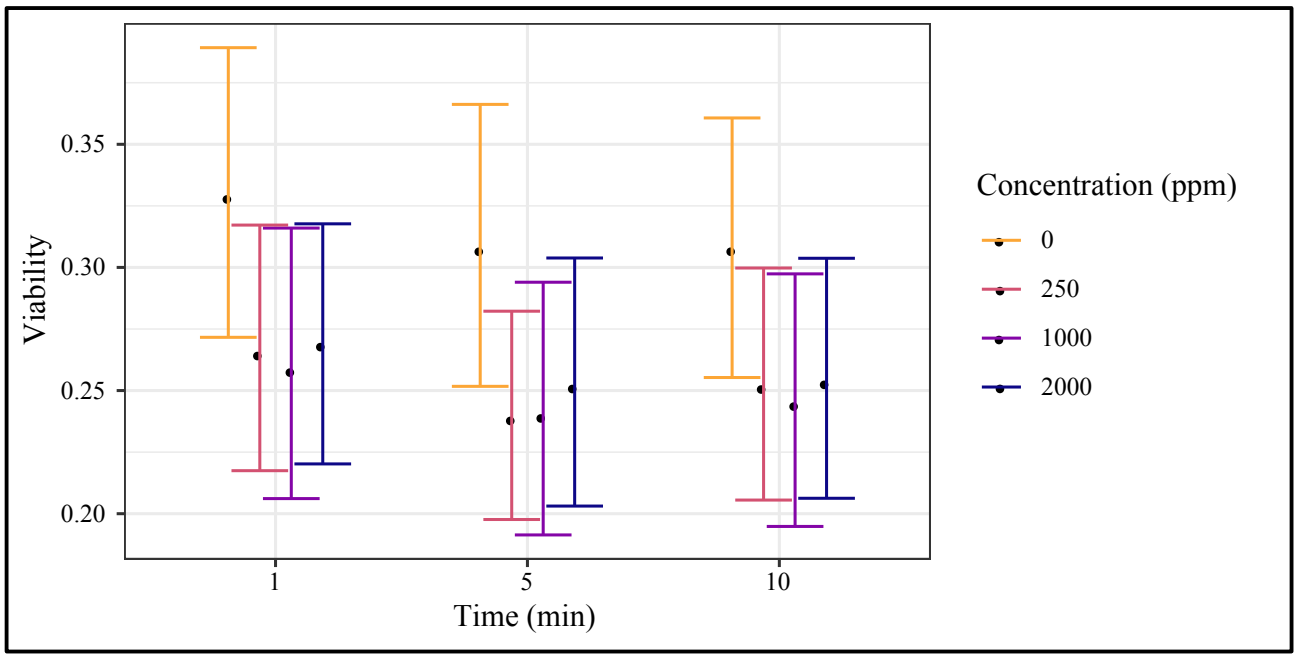

Figure 5.3: *Bonamia exitiosa* cell viability after exposure to seawater control (0 ppm) or free quaternary ammonium (250 ppm, 1,000 ppm and 2,000 ppm) for 1 min, 5 min and 10 min. Bayesian estimated mean, 95% credible intervals.

Table 5.5: Mean *Bonamia exitiosa* cell viability (%) and treatment efficacy (%) per time (1 min, 5 min and 10 min) and concentration treatment of free quaternary ammonium (QA) (250 ppm, 1,000 ppm and 2,000 ppm) or the seawater (0 ppm) control for the QAC assessment.

Time (min)	QA concentration (ppm)	Mean cell viability (%) †	Treatment efficacy (%)
1	**0**	32.88 ± 1.82	0.00
	250	26.27 ± 2.13	20.11
	1,000	25.87 ± 2.70	21.30
	2,000	26.72 ± 2.03	18.74
5	**0**	30.70 ± 0.38	6.62
	250	24.06 ± 1.48	26.81
	1,000	24.13 ± 1.18	26.60
	2,000	25.43 ± 1.38	22.66
10	**0**	30.94 ± 2.11	5.91
	250	25.16 ± 1.43	23.49
	1,000	24.57 ± 0.83	25.27
	2,000	25.67 ± 1.13	21.93

† Cell viability values were mean ± SE; n = 3.

5.4.4 Iodine assessment

In the best model fit for the iodine assessment there was no interaction between time and concentration (Table 5.2). *Bonamia exitiosa* cell viability did not differ between seawater and sodium thiosulfate controls and both controls had higher *B. exitiosa* cell viability than all iodine treatments >1,000 ppm (Figure 5.4). All iodine treatments >2,000 ppm had overlapping credible intervals and had lower *B. exitiosa* cell viability than treatments <1,000 ppm (Figure 5.4). 100% efficacy was achieved in all treatments >2,000 ppm iodine (Figure 5.4, Table 5.6).

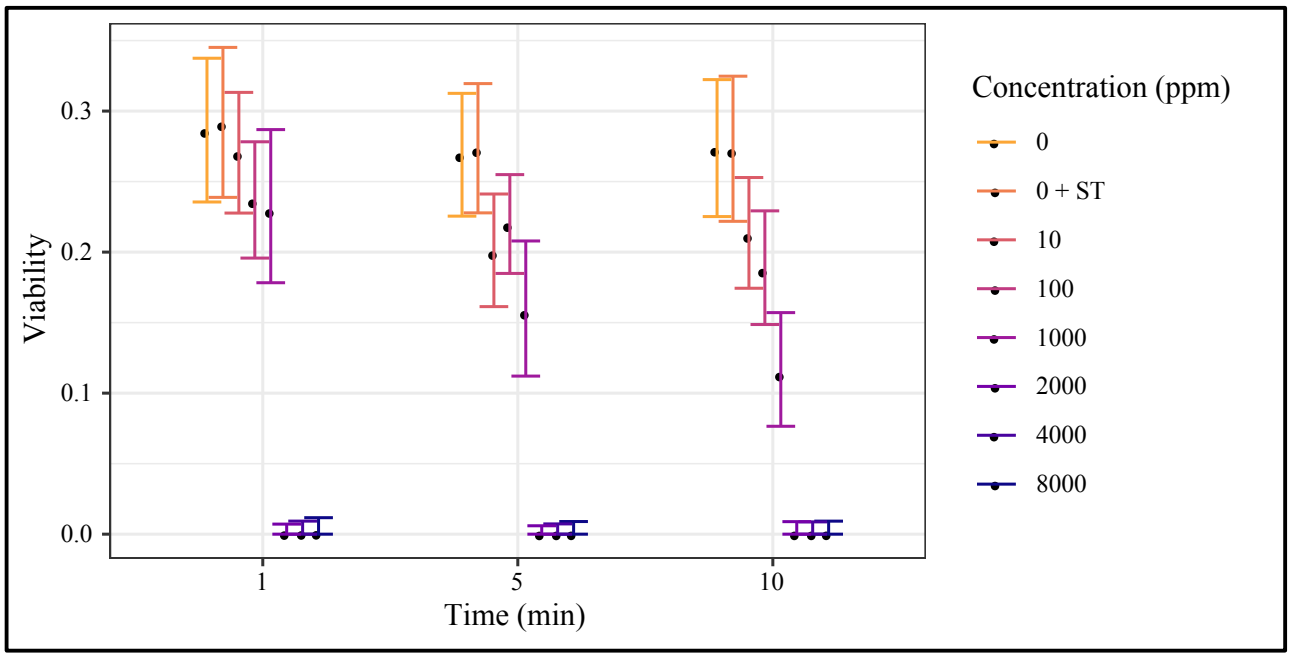

Figure 5.4: *Bonamia exitiosa* cell viability after exposure to seawater control (0 ppm), sodium thiosulfate control (0 ppm + ST) or free iodine (10 ppm, 100 ppm, 1,000 ppm, 2,000 ppm, 4,000 ppm, 8,000 ppm) for 1 min, 5 min and 10 min. Bayesian estimated mean, 95% credible intervals.

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Table 5.6: Mean *Bonamia exitiosa* cell viability (%) and treatment efficacy (%) per time (1 min, 5 min and 10 min) and concentration treatment of free iodine (10 ppm, 100 ppm, 1,000 ppm, 2,000 ppm, 4,000 ppm and 8,000 ppm), the sodium thiosulfate (ST) or the seawater (0 ppm) controls for the iodine assessment.

Time (min)	Iodine concentration (ppm)	Mean cell viability (%) †	Treatment efficacy (%)
1	**0**	29.02 ± 1.47	0.00
	0 (ST)	29.00 ± 2.61	0.10
	10	27.10 ± 2.65	6.62
	100	23.78 ± 2.67	18.07
	1,000	22.50 ± 1.34	22.47
	2,000	0.00 ± 0.00	100.00
	4,000	0.00 ± 0.00	100.00
	8,000	0.00 ± 0.00	100.00
5	**0**	26.87 ± 0.58	7.41
	0 (ST)	27.45 ± 0.70	5.43
	10	19.86 ± 1.58	31.58
	100	21.54 ± 1.79	25.77
	1,000	15.80 ± 0.57	45.57
	2,000	0.00 ± 0.00	100.00
	4,000	0.00 ± 0.00	100.00
	8,000	0.00 ± 0.00	100.00
10	**0**	27.41 ± 1.09	5.56
	0 (ST)	27.25 ± 0.47	6.11
	10	21.14 ± 0.53	27.16
	100	18.74 ± 0.91	35.42
	1,000	11.67 ± 1.67	59.80
	2,000	0.00 ± 0.00	100.00
	4,000	0.00 ± 0.00	100.00
	8,000	0.00 ± 0.00	100.00

† Cell viability values were mean ± SE; n = 3.

5.5 Discussion

Chlorine and iodine disinfectants achieved 100% efficacy against *B. exitiosa* (Tables 5.4 & 5.6). Chlorine is effective against other protozoa; for *Perkinsus olseni* Lester & Davis, 1981, 100% efficacy against zoospores was achieved after exposure to 50 ppm chlorine for 1 h (Casas, Villalba, & Reece, 2002). For *P. olseni* prezoosporangia, Casas et al. (2002). found that 200 ppm chlorine for 1 h was required to achieve 100% efficacy but Goggin, Sewell and Lester (1990) found that 6 ppm chlorine for 30 min was 100% effective. In both cases, however, the minimum effective dose was not determined. We found that 10 min exposure to 40,000 ppm chlorine was required for effective *B. exitiosa* decontamination (Table 5.4). Iodine has variable efficacy against protozoa; 13–18 ppm iodine for 20 min was 100% effective against *Giardia muris* Filice, 1952 cysts but was not effective against *Cryptosporidium* sp. oocytes even after 4 h exposure (Gerba, Johnson, & Hasan, 1997). We found that 10 ppm iodine for 1 min was <6.62% effective against *B. exitiosa* and to achieve 100% efficacy >2,000 ppm for 1 min exposure was required (Table 5.6). There is a large decrease in cell viability between the 1,000 ppm and 2,000 ppm iodine treatments (Figure 5.4); a finer scale iodine analysis between 1,000 ppm and 2,000 ppm would determine if 100% efficacy is also reached at lower iodine concentrations. For exposures >1 min and <10 min, effective *B. exitiosa* decontamination was more dependent on dose than duration of treatment (Figures 5.2 & 5.4). Disinfectants degrade rapidly and shorter decontamination times are preferred to minimise loss of disinfectant and to expedite business operations (DAFF, 2008). Longer exposures to lower chlorine or iodine doses may also be effective against *B. exitiosa* but are more logistically complex to administer.

Viability of control *B. exitiosa* cells varied in the chlorine ranging study, where the 10 min control had lower *B. exitiosa* cell viability than the 1 min control, but the controls did not differ over time for all other assessments. Purified *B. exitiosa* cells display decreasing viability over time, but the largest decreases occur between 24 h and 48 h (Diggles & Hine, 2002) and we assessed all treatments within 24 h of purification.

The QAC disinfectant did not achieve 100% efficacy against *B. exitiosa* in any dose-time combination, nor did *B. exitiosa* viability differ between QAC disinfectant treatments (Figure 5.3, Table 5.5). The lack of disinfectant efficacy of the QAC product does not mean that this product is not useful on oyster farms. QAC products have low toxicity (Gerba, 2015) and are commonly used for cleaning rather than disinfection (DAFF, 2008). Cleaning is a significant component (>90%) of successful decontamination, and must precede disinfection (DAFF, 2008). Organic material can rapidly decrease the efficacy of hypochlorite or iodine disinfectants (Rutala & Weber, 2017; Wild, 2017), and for successful decontamination of *B. exitiosa,* QAC can be used as a cleaning agent prior

to disinfection to remove organic material from equipment. QAC is 100% effective against ostreid herpes virus-1 (OsHV-1) when applied at 2,000 ppm for 10 min (Hick, Evans, Looi, English, & Whittington, 2016) as directed in APVMA PER82160. If PER82160 is amended to include *B. exitiosa*, the amended permit should outline that QAC is not an effective decontaminant for *B. exitiosa*.

Determining the suitability of a disinfectant requires consideration of efficacy, availability, cost, safety to personnel and the environment. Iodine, chlorine and QAC products were assessed against these criteria by the APVMA prior to issue of the MUPs. DAFF (2008) described these disinfectants as having good (chlorine) or acceptable (QAC and iodine) environmental risk profiles, but these products are not intended for release to the marine environment. Iodine, chlorine and QAC products used in this study have the added benefits of being inexpensive (under $200 AUD for 25 L) and readily available. The iodine and chlorine disinfectants have similar cost and are ~1.5 times more expensive than QAC. Using doses that achieve 100% efficacy against *B. exitiosa*, iodine is more economical than chlorine: 1 L of Agridyne can make 8 L of decontamination solution, but 1 L of NaOCl make only 3 L of decontamination solution. Volume economical products can be more practical on farms because less storage space is required.

In Australia, PER14029 authorises use of 10,000 ppm chlorine and PER82160 authorises use of 1,000 ppm iodine, but these concentrations are not effective against *B. exitiosa* (Tables 5.4 & 5.6). Higher concentrations of iodine (>2,000 ppm) and chlorine (10,000 ppm) are required for efficacy against *B. exitiosa* (Tables 5.4 & 5.6). The APVMA permits will therefore require amendment of dose-duration recommendations to provide guidance for decontamination of *B. exitiosa.* Data from this study can inform regulatory authorities in other countries. Effective doses of iodine and chlorine for decontamination of *B. exitiosa* should be trialled against *B. ostreae*. *Bonamia ostreae* is exotic to Australia, but is established in New Zealand (Lane, Webb, & Duncan, 2016) and is a risk to Australian oysters (Buss, Harris, Tanner, Wiltshire, & Deveney, 2020a, Chapter 3). If the concentration-duration criteria for iodine and chlorine we identified for *B. exitiosa* are also effective for decontamination of *B. ostreae*, regulatory authorities could provide directions for use that are effective against both notifiable *Bonamia* species. Such measures would aid management in areas affected by *B. exitiosa* and *B. ostreae*.

Hypochlorites are favoured disinfectants because they break down rapidly (Barnes & Greive, 2013), do not leave toxic residues and are not affected by water hardness, but are corrosive (>500 ppm) and should not be used with metal equipment or containers (Rutala & Weber, 2017). This is a disadvantage for practical application because typical oyster equipment is metal and plastic (plastic mesh, plastic basket frames and metal or plastic basket clips). Chlorine is affected by ammonia and

can have its biocidal activity reduced by formation of slow acting chloramines (Black et al., 1959). Iodine is more stable in the presence of organic compounds than chlorine (Punyani, Narayana, Singh, & Vasudevan, et al., 2006), reacts slowly compared to chlorine and other halogens and is not affected by ammonia (Ellis & Van Vree, 1989; Marks & Strandskov, 1950). Iodine is therefore more suitable than chlorine to use for decontaminating oyster equipment with biofouling or organic sediment deposits. Chlorine has a narrow pH window (pH 4–7) in which it is biocidal (Black et al., 1959). Iodine is less affected by pH than chlorine; its capacity as a disinfectant is only diminished above pH 9 (Kramer, Moore, & Ballinger, 1952; NRC, 1980; Punyani et al., 2006). Seawater has a pH of 8.1 (Clarke et al., 2015) and is suitable for diluting iodine disinfectants for use but diluting with seawater decreases the efficacy of chlorine as a biocide (Black et al., 1959). Chlorine is therefore more suitable for decontamination in freshwater or where freshwater is available. Flexibility in dilutant water improves practicality of iodine use on farms. QACs, like iodine are not severely impacted by organic matter (DAFF, 2008) and are favoured for their safety (Wild, 2017) and stability between pH 4–10 (DAFF, 2008). These properties make iodine the most flexible and suitable choice for decontamination of *B. exitiosa.*

100% efficacy against *B. exitiosa* was achieved using 2,000 ppm iodine for 1 min and 40,000 ppm chlorine for 10 min. QAC is not effective against *B. exitiosa* but is a useful cleaning agent with few workplace safety risks. Iodine is more suitable than chlorine for decontamination of *B. exitiosa*; it has high efficacy, is volume economical and is more stable in the presence of organic matter. These data can be used to inform regulatory approvals and as guidance for decontaminating *B. exitiosa.* Implementation of good decontamination practices will improve mollusc farm biosecurity.

Chapter 6 : General Discussion

Figure 6: *Ostrea angasi* juveniles after three months on an intertidal culture system, Coffin Bay, South Australia.

6.1 Overview

Bonamia exitiosa Hine, Cochennac & Berthe, 2001 was first detected in South Australian Native Oysters (*Ostrea angasi* Sowerby, 1871) using the Bo-Boas/OIE PCR test (OIE, 2019a) and Sanger sequencing of product in 2015 (see Bradley, 2019). *Ostrea angasi* samples from across southern Australia were then genome sequenced and confirmed to be *B. exitiosa* (Bradley, 2019). Beyond this positive diagnosis, there was no information on *B. exitiosa* infection in *O. angasi*. Understanding pathogen transmission, diagnostic test accuracy, short-term and long-term host interactions and decontamination efficacy would aid the oyster industry develop management strategies for *B. exitiosa*. There are 4040 *Bonamia* spp. references in Google Scholar predominantly from Europe, America and New Zealand that outline a range of host- geographic and other patterns of infection and disease, therefore it was likely that many of these had low relevance for Australian *B. exitiosa* infections.

My research provides the first knowledge about *B. exitiosa* epidemiology in *O. angasi* including diagnostic test accuracy, prevalence and intensity, time to first infection and reliable methods to decontaminate *B. exitiosa.*

6.2 Disease surveillance

The role of disease surveillance is to prove freedom from disease for an area or to progress disease control strategies including containment and asset protection (Peeler & Taylor, 2011). The ability of a test to accurately detect a pathogen depends on the DSe and DSp of the test (Weinstein, Obuchowski, & Lieber, 2005). Prior to my study, diagnostic tests for *Bonamia* spp. were not validated with Bayesian methods and were only validated for other pathogen-host systems including *Bonamia ostreae* Pichot, Comps, Tigé, Grizel & Rabouin, 1980 in *Ostrea edulis* Linnaeus, 1758 (see Carnegie, Barber, Culloty, Figueras, & Distel, 2000; Flannery et al., 2014; Lynch, Mulcahy & Culloty, 2008; Marty et al., 2006; Robert et al., 2009) or *B. exitiosa* infection in *Ostrea chilensis* Philippi, 1844 (see Diggles, Cochennec-Laureau & Hine, 2003; Michael, Forman, Hulston, Fu & Maas, 2015), but there were no diagnostic data for *B. exitiosa* in *O. angasi*. Predictive values are the probability of an organism having disease after a positive test result (positive predictive value, PPV) and not having disease after a negative test result (negative predictive value, NPV); these are calculated using the DSe and DSp (Fegan, 2000). PPV and NPVs inform the clinical relevance of the test and are prevalence dependent. Prevalence varies between host populations, geographically, and with a range of other influences. For different oyster populations PPV and NPV will therefore differ and influence the accuracy of diagnoses. DSe and DSp are influenced by disease intensity; if there is higher intensity of infection, sensitivity increases

as there is a higher chance of making a positive diagnosis (Medeiros, Zangwill, Bowd, Sample & Weinreb, 2006; Parikh, Mathai, Parikh, Sekhar & Thomas, 2008). Farmed *O. angasi* have low *B. exitiosa* intensity (Chapters 2 and 4) and this differs from many *Bonamia* spp. infections in Europe and New Zealand (Corbeil, Handlinger & Crane, 2009; Culloty, Cronin & Mulcahy, 2004). Test performance is therefore influenced by both prevalence and intensity changes and justifies assessing *B. exitiosa* tests in a new host.

I surveyed three South Australian farming regions and determined the DSe and DSp of heart smear, histology and qPCR using Bayesian methods which assume that tests are not perfect (Chapter 2). When assessing diagnostic tests, it is important to choose a population that has or reflects natural infection dynamics for a disease (Laurin et al., 2018). To minimise bias for test results, diagnostic tests were used on farmed *O. angasi* populations that were naturally infected with *B. exitiosa* in the field. If diagnostic tests were assessed using an *O. angasi* population infected by other means, such as laboratory inoculation, it would have provided estimates of DSe and DSp that may not be applicable for practical use in the field. Diagnostic test information provided in Chapter 2 informed research design for all subsequent chapters in this thesis and provided me with reliable estimates of prevalence and in turn reliable interpretation of *B. exitiosa* test results. DSe and DSp data (Chapter 2) can be used as a basis for survey design in Australia and for future *B. exitiosa* research to calculate surveillance or experimental sample sizes when using each test or test combination and provide better estimates of prevalence.

I found that *B. exitiosa* infection in South Australian farmed *O. angasi* populations is characterised by high prevalence and low intensity, but the *O. angasi* industry is still developing and most farms culture *C. gigas*. While clinical disease has not yet been reported for South Australia, confirmation of *B. exitiosa* infection (Chapter 2) means if the industry expands, the risk that clinical *B. exitiosa* infections will develop in *O. angasi* populations is high. Understanding *B. exitiosa* prevalence and intensity informs farmers about disease risk and provides a basis for developing a management plan for *B. exitiosa*.

The purpose of surveillance is the most important component of choosing a diagnostic test; tests with high DSe have low false negatives and are better to prove freedom for a population because they provide improved reliability in negative test results. Tests with high DSp have low false positive rates and are better for assessing prevalence because they provide better reliability for positive test results (Fegan, 2000). I found histology was the best single test for *B. exitiosa* with highest DSe (0.76) and DSp (0.93) (Chapter 2). I also found using tests in combination and defining an infected case as one where either test was positive (OR-rule) had higher DSe and NPV and was better for proving freedom than single tests which had lower DSe (Chapter 2). For assessing

prevalence however, single qPCR or histology tests were as good as tests in combination if an infected case was defined as one where both tests were positive (AND-rule), due to high DSp and PPV (Chapter 2). Using histology and qPCR in the OR-rule maximised both DSe and DSp and is suitable for assessing prevalence and/or proving freedom (Chapter 2). *Bonamia exitiosa* is widespread in Australian oyster farms (Chapters 2 and 4) and therefore assessing prevalence may be more important than proving freedom for a population. Prevalence is the preferred parameter to measure in population surveillance to understand infection dynamics and disease risk. Site-specific prevalence data can assist farmers select sites; preferred culture sites would have low *B. exitiosa* prevalence that increases slowly over the culture cycle. To measure prevalence, qPCR provides rapid results and can exclude pathogens that are morphologically similar compared to histology (Stokes & Burreson, 2001). Intensity is a better attribute to measure, however, when monitoring disease dynamics and pathological changes over time. Intensity provides additional information about the effect of a pathogen on individual hosts and forewarns the development of clinical disease in a population. Unlike qPCR, histology can accurately estimate *B. exitiosa* intensity and prevalence and can also provide additional information on oyster condition and confirm pathogen viability (Aranguren & Figueras, 2016; Diggles et al., 2003). Surveillance for *B. exitiosa* in Australia should use qPCR for surveying new regions, histology for monitoring infection dynamics over time, or a combination of histology and qPCR in the OR-rule to accommodate both scenarios (Chapter 2). The use of both microscopy and molecular tests in combination provides information on pathogen viability and DNA presence which is preferable when confirming disease (Aranguren & Figueras, 2016; Burreson & Ford, 2004). While heart smears had lower DSe and DSp than histology and qPCR, heart smears are cost-effective and rapid and, like histology, provide a measure of intensity (Diggles et al., 2003). Understanding heart smear DSe and DSp improved reliability of prevalence estimates from heart smears and can therefore provide a reliable prediction of *B. exitiosa* infections in a population.

Understanding which pathogen is present is also important for test consideration. *Bonamia exitiosa* and *B. ostreae* both occur in New Zealand (Lane, Webb, & Duncan, 2016), in Spain (Abollo et al., 2008) and in the USA (Engelsma, Culloty, Lynch, Arzul & Carnegie, 2014; Hill et al., 2014). There are some morphological differences that can be used to differentiate *B. ostreae* and *B. exitiosa*; *Bonamia ostreae* cell are smaller (0.9–2 µm in diameter) with an eccentric nucleus, whereas *B. exitiosa* cells are larger (1.5–4 µm in diameter), with a concentric nucleus (Lane et al., 2016). A trained pathologist can discern differences between *Bonamia* spp., however for an oyster population where only one *Bonamia* sp. has been detected, identifying infections as caused by a different species or that a coinfection had emerged is difficult (Abollo et

al., 2008). Molecular tests including ISH and PCR improve species specificity of surveillance if tests are designed to differentiate species. I used a *Bonamia* qPCR (Corbeil et al., 2006) that does not differentiate species, but provides a positive to all described *Bonamia* spp. *Bonamia ostreae* poses a risk to Australia but test performance has been assessed only for *B. exitiosa* (Chapter 2). Multiplex PCR is a strategy that can combine tests for *B. exitiosa* and *B. ostreae* and is feasible (Ramilo, Navas, Villalba, & Abollo, 2013). Such assays could provide a means for investigating *Bonamia* spp. outbreaks in Australia where species differentiation is necessary, but validation of these assays is required.

6.3 *Bonamia* transmission

Infection dynamics vary between different *Bonamia* spp. Time to first infection is >2 months for *B. ostreae* infections in *O. edulis* (see Culloty & Mulcahy, 1996; Elston, Farley & Kent, 1986; Lallias et al., 2008; Lynch, Armitage, Wylde, Mulcahy & Culloty, 2005; Montes, 1991; Montes, Ferro-Soto, Conchas & Guerra, 2003), whereas time to first *B. exitiosa* infection in *Crassostrea ariakensis* Fujita, 1913 is >14 days exposure in Bogue Sound (Audemard, Carnegie, Hill, Peterson & Burreson 2014). Prior to my study, *B. exitiosa* infection dynamics in *O. angasi* were unknown. I found that *B. exitiosa* causes rapid and lethal infection in juvenile *O. angasi*; time to first infection was under 10 days and the first mortality occurred at 12 days (Chapter 3), which is faster than described for any *Bonamia* spp.-host system worldwide. My infection model shows that extrinsic factors are likely drivers of *Bonamia* spp. infections (Chapter 2) and other *Bonamia* spp.-host systems may also have faster times to first infection if infection pressure within the system is higher. Time to first *Bonamia* spp. infection is therefore dependent largely on intensity and duration of exposure and host species.

In evolutionary terms, *B. exitiosa* is most likely a recent introduction to South Australia (Bradley, 2019). Although the route of introduction is unknown, it is likely that it was brought to South Australia via an anthropogenic route. South Australian *O. angasi* populations have not been exposed to this pathogen for a sufficient period of time for resistance to have emerged broadly through mass selection. *Ostrea edulis* has been recorded in Western Australia (Morton, Lan, & Slack-Smith, 2003), but a broad understanding of Australian flat oyster populations is lacking. It would be useful to use molecular approaches to assess the genetic structure of *Ostrea* spp. populations Australia-wide to understand the phylogeography of the group. Understanding host phylogeny may help to explain the sometimes disparate impacts of *B. exitiosa* and identify markers that could be used to identify families for a breeding program for resistance against *B. exitiosa*.

CHAPTER 6: GENERAL DISCUSSION

I showed that *B. exitiosa* prevalence of infection increases in naïve animals over time in the laboratory (Chapter 3) and in the field (Chapter 4) and that clinical infection can develop very rapidly (<10 days) in the laboratory (Chapter 3). While *Bonamia* sp. epizootics in *O. angasi* have been recorded in Victoria, Western Australia and Tasmania (Hine & Jones, 1994), increasing prevalence on farm (Chapter 4) and rapid clinical progression in environments with high infection pressure (Chapter 3) mean future epizootics are likely wherever the *O. angasi* industry expands; increased oyster density from industry expansion would increase *B. exitiosa* infection pressure in a farming region. South Australian growers have already observed lower survival in *O. angasi* than for *Crassostrea gigas* Thunberg, 1793 (unpublished data), indicating that some *Bonamia*-related mortality may already have occurred. Ill-suited culture systems for *O. angasi* also contribute to lower survival; *O. angasi* are a sub-tidal oyster (Edgar, 2008) that is frequently grown in intertidal culture systems. *Crassostrea gigas* is a likely host of *Bonamia* spp. (see Diggles, 2003; Lynch et al., 2010) and given that it is the most farmed oyster species in Australia (Nell, 2001), its role in *B. exitiosa* transmission to *O. angasi* requires further research.

Bonamia exitiosa and *B. ostreae* are haplosporidians, but they do not have a spore in their lifecycle and appear to transmit directly without an intermediate host (Arzul & Carnegie, 2015; Carnegie et al., 2006). I demonstrated that direct infection occurs for *B. exitiosa* between live *O. angasi* (Chapter 3). The absence of an intermediate host is difficult to prove and cannot conclusively be discounted (Arzul & Carnegie, 2015; Lynch, Armitage, Coughlan, Mulcahy and Culloty, 2007). *Bonamia ostreae* has been detected in zooplankton by PCR, but this method cannot differentiate *B. ostreae* cells in water from infected zooplankton. While infection of atypical hosts has been documented (Lynch, Armitage, Wylde, Mulcahy, & Culloty, 2006; Lynch et al., 2007), the ability of atypical hosts to transmit *Bonamia* is unknown. While an intermediate host and/or spores are possible for *B. exitiosa*, management should assume live oysters in close proximity can readily spread infection without the need for other hosts or lifecycle phases.

I found that larger *O. angasi* were associated with higher *B. exitiosa* intensity and showing infections consistent with clinical disease including decreased meat:shell ratio (Chapter 2). Clinical disease with concomitant high intensity has been reported in *B. ostreae* infecting *O. edulis* (see Arzul et al., 2011; Culloty & Mulcahy, 1996; Robert, Borel, Pichot & Trut, 1991) and for *B. exitiosa* in *O. chilensis* (see Cranfield, Dunn, Doonan & Michael, 2005; Hine, 1996). The market for *O. angasi* in Australia is not well established but has potential for expansion. *Ostrea angasi* spawn when they are over two years of age (Crawford, 2016) but spawning stress has been linked to increased *B. exitiosa* intensity and prevalence in the gonad of *O. chilensis* (see Hine, 1991). Older *O. angasi* that encounter spawning stress may therefore have higher *B. exitiosa* intensity and

prevalence than immature juvenile oysters. I found that after one year exposure on farm, intensity remained low and oysters had not developed clinical disease but oysters had also not encountered spawning stress (Chapter 4). Ben-Horin et al. (2018) used modelling that predicted total parasite load in the environment could be decreased if farmed shellfish are harvested prior to development of clinical infection and parasite shedding. Decreasing parasite load in the environment would have the flow-on effect of decreasing disease impacts on wild oyster populations (Ben-Horin et al., 2018). *Ostrea angasi* reef restoration projects have commenced in Australia (Gillies, Crawford & Hancock, 2017) and will increase population densities of wild *O. angasi*.

Increased oyster densities are associated with increased *Bonamia* spp. prevalence (Arzul & Carnegie, 2015; Cranfield et al., 2005; Lallias et al., 2008). If older oysters on farm are more prone to clinical disease and mortality from *B. exitiosa*, establishing markets for smaller and younger oysters is a strategy to manage *B. exitiosa* infection and decrease the likelihood that industry expansion and reef restoration projects could drive epizootic events. Market development should therefore consider an oyster size that can be cultured and harvested within a timeframe shorter than that in which *B. exitiosa* clinical disease develops on farms.

Farm sites with low *B. exitiosa* prevalence are preferred over areas with high prevalence; lower density of infected individuals decreases the rate of clinical disease development (Cranfield et al., 2005). Higher prevalence means higher pathogen availability and higher risk of pathogen shedding, therefore an increased chance for infection to spread. I found that within three months all surveyed farm sites were positive for *B. exitiosa* infection, but Cowell and Streaky Bay took nine months to reach >0.50 prevalence whereas Coffin Bay sites took six months (Chapter 4). Streaky Bay and Cowell are therefore potentially more appropriate for farming *O. angasi* than Coffin Bay. After one year Cowell had lower *B. exitiosa* prevalence (0.57) than at other sites (>0.78) (Chapter 4). To aid selecting better sites with lower likelihood of clinical disease development, deployment of limited numbers of susceptible sentinel oysters and monitoring their *Bonamia* status is therefore recommended as a surveillance strategy prior to farming in a new area.

Seasonal patterns of *B. exitiosa* intensity were inconsistent between sites, but higher intensity was associated with animals in poor condition (lower meat:shell ratio) in autumn and winter and with animals in better condition (higher meat:shell ratio) in spring and summer (Chapter 4). Poor condition and high *Bonamia* intensity are associated with oysters undergoing mortality (Corbeil et al., 2009; Rogan, Culloty, Cross & Mulcahy, 1991). Hine (1991) found that *O. chilensis* have light *B. exitiosa* infections in spring and summer and heavier infections in winter. I also found that older and larger oysters sampled in winter had highest *B. exitiosa* intensities (Chapter 2). Southern hemisphere *B. exitiosa* infections in *O. angasi* may therefore have similar seasonal patterns to

infections in *O. chilensis* and a long-term seasonal study in *O. angasi* would provide further support of southern hemisphere seasonal similarities.

Subsequent to the description of *B. ostreae* in 1979 (Arzul et al., 2006), there has been substantial research on *Bonamia* spp. (see reviews: Arzul & Carnegie, 2015; Burreson & Ford, 2004; Carnegie & Cochennec-Laureau, 2004) but little progress has been made in developing practical solutions for farm management and improving production. Globally, industries are still suffering decreased productivity; between 1986–1992 an epizootic of *B. exitiosa* in the New Zealand Foveaux Strait *O. chilensis* fishery decreased populations to <10% pre-disease biomass and caused a fishery closure (Cranfield et al., 2005). The Foveaux Strait fishery population is driven by mortality from *B. exitiosa* and the population has never recovered to pre-outbreak biomass (Fu, Dunn, Michael, & Hills, 2016). After *B. ostreae* epizootics, French *O. edulis* production decreased from its industry maximum of 28,000 tonnes in 1960 to below 2,000 tonnes in 1979 and never recovered (Buestel, Ropert, Prou & Goulletquer, 2009). Since the *B. ostreae* epizootic in *O. edulis*, *C. gigas* are the main oyster species cultivated in France (Buestel et al., 2009). *Bonamia* spp. have therefore had significant negative implications on oyster industries worldwide.

Access to *B. ostreae* resistant stock provides a functional management strategy for oyster farmers, but appears not to have been implemented widely. Programs that selectively breed *O. edulis* for resistance against *B. ostreae* have produced resistant stock in France (Naciri-Graven, Martin, Baud, Renault, & Gèrard, 1998) and Ireland (Lynch, Flannery, Hugh-Jones, Hugh-Jones & Culloty, 2014). Oyster breeding programs use best linear unbiased prediction analysis on oyster families to produce estimated breeding values to select for different traits (Camara & Symonds, 2014). The Australian *O. angasi* industry is small and emerging, with no breeding program. A breeding program selecting for beneficial traits would aid development and sustainable expansion of the Australian *O. angasi* industry and provide *Bonamia* spp. resistant stock for reef restoration. While disease resistance to *B. exitiosa* would be beneficial, other traits to select for could include growth, spawning time or to improve oyster resilience against changes in climate such as ocean acidification or temperature tolerance. *Ostrea angasi* are sequential protandric hermaphrodites that brood larvae for 15–22 days and this makes breeding families difficult; to cross individuals, two *O. angasi* are placed in aquaria until larvae are released and this process is time consuming and costly (O'Connor, 2015). While breeding *O. angasi* for resistance may provide a management solution for managing *B. exitiosa* in Australia, increased industry interest and a cost-benefit analysis and are needed to justify commencement of a breeding program. A breeding program would bring mutual benefits to aquaculture industries and reef restoration and funds could be pooled between industries for a breeding program. I developed an infection model (Chapter 3) that provides a means

to challenge *O. angasi* with *B. exitiosa* infection which could facilitate selection for *B. exitiosa* in a breeding program.

6.4 Decontamination

The purpose of using disinfectants in aquaculture is to improve farm biosecurity by reducing pathogen loads and spread. Effective decontamination with disinfectants can reduce risk of translocating equipment, machinery and personnel between infected and non-infected zones (DAFF, 2008).

In Australia, two minor use permits (MUPs) issued by the Australian Pesticides and Veterinary Medicines Authority (APVMA) provide regulatory authority for use of decontaminants for the Australian oyster industry. PER14029 is a disinfection permit to use sodium hypochlorite (NaOCl) against all oyster pathogens and PER82160 permits use of an iodine based disinfectant (Agridyne), a sodium hydroxide based disinfectant (Solo-plus), a QAC (Detsan), and disinfectants containing potassium peroxomonosulphate triple salt, sodium dodecyl benzene sulphonate and sodium chloride (Virkon S) against ostreid herpesvirus-1 microvariant (OsHV-1 microvariant). Decontamination guidelines for *B. exitiosa*, however, were lacking. I tested Detsan, NaOCl and Agridyne, currently permitted by APVMA for use on oyster equipment against *B. exitiosa* and found Agridyne and NaOCl to be 100% effective (Chapter 5). To decontaminate *B. exitiosa* growers can use 2,000 ppm free iodine for 1 min (125 mL Agridyne per 1 L of water) or 10,000 ppm free chlorine (320 mL NaOCl (12.5% available chlorine) per 1L of water) to bath oyster equipment prior to stocking naïve *O. angasi* spat on farm or moving between zones. In laboratories, iodine and chlorine can be used to decontaminate laboratory glassware, surgical tools and other equipment that has come into contact with oyster material infected with *B. exitiosa.* Iodine is preferred over chlorine for decontamination because iodine is less impacted by organic material (Punyani, Narayana, Singh & Vasudevan, 2006) (Chapter 5). Oyster equipment typically has high biofouling and attached sediment and choosing disinfectants that are not impeded by organic matter is important. Equipment should be cleaned before decontamination because cleaning is responsible for >90% effective decontamination (DAFF, 2008). Detsan was not effective at decontaminating *B. exitiosa*, but can be used as a cleaning agent prior to disinfection (Chapter 5) and is favoured for its safety, particularly on human skin (DAFF, 2008). QACs are effective against other pathogens; 10 min exposure to 2,000 QAC has 100% efficacy against OsHV-1 (Hick, Evans, Looi, English & Whittington, 2016) and this dose-time treatment is specified in PER82160. Detsan is therefore a useful disinfectant or cleaning product to use on an oyster farm.

Both APVMA MUPs for disinfecting oyster equipment will need amendment to incorporate patterns of use for *B. exitiosa*. *Bonamia ostreae* is also a risk for Australian *O. angasi* (see Animal Health Committee, 2018) and disinfectant treatments that are effective against *B. exitiosa* should be trialled against *B. ostreae*.

6.5 Future research

The effect of pooling samples, particularly understanding DSe and DSp for pooled qPCR should be examined; Lane, Jones and McDonald (2017) assessed the analytical limit of detection for pooled *B. ostreae* PCR, but DSe and DSp were not determined. Understanding diagnostic performance of pooled samples analysed by PCR could provide a cost-effective means of surveillance. Bayesian LCMs provide a reliable method for comparing tests and form a statistical basis for epidemiological research.

Breeding programs can use family lines and selection to breed stock with different beneficial traits. I have created an infection model that can test susceptibility of *O. angasi* family lines to *B. exitiosa* infection.

Monitoring *B. exitiosa* infection through consecutive years and in multiple age classes would improve knowledge of seasonal dynamics and patterns over time. A long-term seasonal study would provide further support of southern hemisphere seasonal similarities for *B. exitiosa* infection. Understanding *B. exitiosa* infection dynamics in farmed *O. angasi* for a full grow-out period would inform management strategies including timing harvest to avoid development of clinical infection and parasite shedding from farms and avoid coincident stressors associated with *Bonamia* infection and spawning. Defining optimal harvest times can help inform a suitable market size for *O. angasi.*

Most southern Australian states practice mixed species oyster farming. *Crassostrea gigas* and *O. angasi* are often co-farmed, and interest in farming *Saccostrea glomerata* Gould, 1850 more broadly across southern Australia is growing (Li, Miller-Ezzy, Crawford, Gardner, & Deveney, 2019). Research into the role of cupped oysters in *B. exitiosa* transmission would aid decisions about importing and growing *S. glomerata* in new areas. Improved understanding inter-species infection dynamics would facilitate better management decisions and placement of oyster species on farms. Surveillance of *O. angasi* in restored reefs would inform understanding of the epidemiological links between farmed and wild populations of *O. angasi*.

I obtained data that facilitates effective decontamination of *B. exitiosa*. These disinfectants should be assessed against *B. ostreae*. Using disinfectants in a way that deactivates multiple pathogens is practical for industries worldwide, particularly where multiple *Bonamia* species occur. Efficacy of other disinfectants against *B. exitiosa* could also be investigated. A greater variety of

available disinfectants would increase choice and provide better products for specific decontaminant uses. Some disinfectants may be easier to use, particularly if existing regulatory authorities can be amended for their use rather than applying for and maintaining a new permit.

6.6 Recommendations for management

1. Australian *O. angasi* aquaculture is a developing industry. *Bonamia exitiosa* occurs in South Australia and expansion of the *O. angasi* industry requires a management framework. A surveillance program should be used to select optimal sites and determine harvest periods and diagnostic test data required are now available for *B. exitiosa* in *O. angasi* to facilitate a surveillance program.
2. Where proving freedom from *B. exitiosa* is required, such as in a hatchery setting, using test combinations in the OR-rule is better than the use of single tests. While all OR-rule test combinations are adequate for proving freedom, histology and qPCR combination in the OR-rule give the highest DSe and NPV. This combination incorporating microscope visualisation and DNA confirmation is also best for assessing parasite intensity and diagnosing disease.
3. qPCR should be used for *B. exitiosa* surveillance in new areas to understand prevalence and histology should be used for monitoring infection dynamics over time including understanding intensity. A combination of histology and qPCR in the OR-rule can be used when a measure of *B. exitiosa* prevalence and intensity are both required. Test DSe and DSp can be used to inform sample size for *B. exitiosa* surveillance and prior test information can inform data analysis and reliable estimates of prevalence.
4. Development of an *O. angasi* breeding program for resistance against *B. exitiosa* with selection of other traits would facilitate industry expansion. Mitigation of *B exitiosa* impacts is in the national interest and there should be national collaboration by industries towards a breeding program. A cost-benefit analysis would help justify the decision.
5. *Ostrea angasi* reefs should be positioned to minimise impacts on oyster farming regions and the potential of farming to decrease environmental loads of pathogens should be investigated.
6. Surveillance should be used to select farming sites with low *B. exitiosa* prevalence and slow prevalence increase over the farming cycle. Cowell or Streaky Bay each are better suited for growing *O. angasi* than Coffin Bay; prevalence was lower and it took longer (>9 months) to high reach prevalence (>0.50) (Chapter 4). Deployment of naïve sentinels

at various farm locations is a management strategy to monitor presence and development of clinical infection over time for that area.

7. Effective decontamination of *B. exitiosa* can be achieved with 2,000 ppm iodine baths for 1 min or 40,000 ppm chlorine baths for 10 min. Decontamination of equipment is recommended prior to stocking spat on farms, moving equipment or personnel between biosecurity zones, or decontaminating hatcheries between breeding runs. The tested QAC was not effective for *B. exitiosa* decontamination, but its benign safety characteristics and efficacy against OsHV-1 make it a useful cleaning agent on oyster farms, for use prior to bathing in either chlorine or iodine for effective *B. exitiosa* decontamination.

6.7 Conclusion

My research provides the first data on *B. exitiosa* infection in *O. angasi*. I have demonstrated the distribution of *B. exitiosa* in South Australia and assessed short-term and long-term infection dynamics. Surveillance programs can be developed using diagnostic performance data from my work and I have provided effective decontaminants for use on farms. The Australian *O. angasi* industry is developing and the information I have provided can inform industry expansion decisions and improve current management practices.

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Appendices

Appendix A: (Chapter 2)

JAGS code for LCM used in Chapter 2 including conditional dependence between tests. Adapted from Lewis and Torgerson (2012).

```
model{
  ## Priors

          ## Sensitivities and specificities
          ## 1. Heart smear, 2. Histology, 3. qPCR
          Se1 ~ dbeta(4,3)
          Sp1 ~ dbeta(4,3)
          Se2 ~ dbeta(4,3)
          Sp2 ~ dbeta(20.1,0.3)
          Se3 ~ dbeta(4,3)
  Sp3 ~ dbeta(20.1,0.3)

  ## Prevalence
  for (j in 1:3) {
          pi[j] ~ dbeta(1,1)
  }

  ## Covariance
  covp12 ~ dnorm(0,9)
  covn12 ~ dnorm(0,9)
  covp13 ~ dnorm(0,9)
  covn13 ~ dnorm(0,9)
  covp23 ~ dnorm(0,9)
  covn23 ~ dnorm(0,9)

  ## Correlation
  rhop12 <- covp12/(sqrt(Se1*(1-Se1)*Se2*(1-Se2)))
  rhon12 <- covn12/(sqrt(Sp1*(1-Sp1)*Sp2*(1-Sp2)))
```

```
rhop13 <- covp13/(sqrt(Se1*(1-Se1)*Se3*(1-Se3)))
rhon13 <- covn13/(sqrt(Sp1*(1-Sp1)*Sp3*(1-Sp3)))
rhop23 <- covp23/(sqrt(Se2*(1-Se2)*Se3*(1-Se3)))
rhon23 <- covn23/(sqrt(Sp2*(1-Sp2)*Sp3*(1-Sp3)))
## Probabilities for each different result
for (i in 1:n) {
        p[i,1] <- pi[site[i]]*((1-Se1)*(1-Se2)*(1-Se3)+covp12+covp13+covp23) + (1-
                pi[site[i]])*((Sp1)*(Sp2)*(Sp3)+covn12+covn13+covn23)  ## 0, 0, 0
        p[i,2] <- pi[site[i]]*((1-Se1)*(1-Se2)*(Se3)+covp12-covp13-covp23) + (1-
                pi[site[i]])*((Sp1)*(Sp2)*(1-Sp3)+covn12-covn13-covn23)  ## 0, 0, 1
        p[i,3] <- pi[site[i]]*((1-Se1)*(Se2)*(1-Se3)-covp12+covp13-covp23) + (1-
                pi[site[i]])*((Sp1)*(1-Sp2)*(Sp3)-covn12+covn13-covn23)  ## 0, 1, 0
        p[i,4] <- pi[site[i]]*((1-Se1)*(Se2)*(Se3)-covp12-covp13+covp23) + (1-
                pi[site[i]])*((Sp1)*(1-Sp2)*(1-Sp3)-covn12-covn13+covn23)  ## 0, 1, 1
        p[i,5] <- pi[site[i]]*((Se1)*(1-Se2)*(1-Se3)-covp12-covp13+covp23) + (1-
                pi[site[i]])*((1-Sp1)*(Sp2)*(Sp3)-covn12-covn13+covn23)  ## 1, 0, 0
        p[i,6] <- pi[site[i]]*((Se1)*(1-Se2)*(Se3)-covp12+covp13-covp23) + (1-
                pi[site[i]])*((1-Sp1)*(Sp2)*(1-Sp3)-covn12+covn13-covn23)  ## 1, 0, 1
        p[i,7] <- pi[site[i]]*((Se1)*(Se2)*(1-Se3)+covp12-covp13-covp23) + (1-
                pi[site[i]])*((1-Sp1)*(1-Sp2)*(Sp3)+covn12-covn13-covn23)  ## 1, 1, 0
        p[i,8] <- pi[site[i]]*((Se1)*(Se2)*(Se3)+covp12+covp13+covp23) + (1-
                pi[site[i]])*((1-Sp1)*(1-Sp2)*(1-Sp3)+covn12+covn13+covn23)  ## 1, 1, 1

                ## Error checking since (0,1) bounds could be exceeded
                checks[i,1]  <- (1-Se1)*(1-Se2)*(1-Se3)+covp12+covp13+covp23
                checks[i,2]  <- (Sp1)*(Sp2)*(Sp3)+covn12+covn13+covn23
                checks[i,3]  <- (1-Se1)*(1-Se2)*(Se3)+covp12-covp13-covp23
                checks[i,4]  <- (Sp1)*(Sp2)*(1-Sp3)+covn12-covn13-covn23
                checks[i,5]  <- (1-Se1)*(Se2)*(1-Se3)-covp12+covp13-covp23
                checks[i,6]  <- (Sp1)*(1-Sp2)*(Sp3)-covn12+covn13-covn23
                checks[i,7]  <- (1-Se1)*(Se2)*(Se3)-covp12-covp13+covp23
                checks[i,8]  <- (Sp1)*(1-Sp2)*(1-Sp3)-covn12-covn13+covn23
                checks[i,9]  <- (Se1)*(1-Se2)*(1-Se3)-covp12-covp13+covp23
                checks[i,10] <- (1-Sp1)*(Sp2)*(Sp3)-covn12-covn13+covn23
```

```
checks[i,11] <- (Se1)*(1-Se2)*(Se3)-covp12+covp13-covp23
checks[i,12] <- (1-Sp1)*(Sp2)*(1-Sp3)-covn12+covn13-covn23
checks[i,13] <- (Se1)*(Se2)*(1-Se3)+covp12-covp13-covp23
checks[i,14] <- (1-Sp1)*(1-Sp2)*(Sp3)+covn12-covn13-covn23
checks[i,15] <- (Se1)*(Se2)*(Se3)+covp12+covp13+covp23
checks[i,16] <- (1-Sp1)*(1-Sp2)*(1-Sp3)+covn12+covn13+covn23

valid[i]<- step(1-p[i,1])*step(p[i,1])*
step(1-p[i,2])*step(p[i,2])*
step(1-p[i,3])*step(p[i,3])*
step(1-p[i,4])*step(p[i,4])*
step(1-p[i,5])*step(p[i,5])*
step(1-p[i,6])*step(p[i,6])*
step(1-p[i,7])*step(p[i,7])*
step(1-p[i,8])*step(p[i,8])*
step(1-checks[i,1])*step(checks[i,1])*
step(1-checks[i,2])*step(checks[i,2])*
step(1-checks[i,3])*step(checks[i,3])*
step(1-checks[i,4])*step(checks[i,4])*
step(1-checks[i,5])*step(checks[i,5])*
step(1-checks[i,6])*step(checks[i,6])*
step(1-checks[i,7])*step(checks[i,7])*
step(1-checks[i,8])*step(checks[i,8])*
step(1-checks[i,9])*step(checks[i,9])*
step(1-checks[i,10])*step(checks[i,10])*
step(1-checks[i,11])*step(checks[i,11])*
step(1-checks[i,12])*step(checks[i,12])*
step(1-checks[i,13])*step(checks[i,13])*
step(1-checks[i,14])*step(checks[i,14])*
step(1-checks[i,15])*step(checks[i,15])*
step(1-checks[i,16])*step(checks[i,16])

L[i]<- equals(valid[i],1)*(
equals(all.tests[i,1],0)*equals(all.tests[i,2],0)*equals(all.tests[i,3],0)*p[i,1] +
```

```
                equals(all.tests[i,1],0)*equals(all.tests[i,2],0)*equals(all.tests[i,3],1)*p[i,2] +
                equals(all.tests[i,1],0)*equals(all.tests[i,2],1)*equals(all.tests[i,3],0)*p[i,3] +
                equals(all.tests[i,1],0)*equals(all.tests[i,2],1)*equals(all.tests[i,3],1)*p[i,4] +
                equals(all.tests[i,1],1)*equals(all.tests[i,2],0)*equals(all.tests[i,3],0)*p[i,5] +
                equals(all.tests[i,1],1)*equals(all.tests[i,2],0)*equals(all.tests[i,3],1)*p[i,6] +
                equals(all.tests[i,1],1)*equals(all.tests[i,2],1)*equals(all.tests[i,3],0)*p[i,7] +
                equals(all.tests[i,1],1)*equals(all.tests[i,2],1)*equals(all.tests[i,3],1)*p[i,8]) +
                (1-equals(valid[i],1))*(1e-16)

                ## ones trick
                pr[i] <- L[i] / 1
                ones[i] ~ dbern(pr[i])
}

 ## Joint test Se and Sp
Se1and2 <- Se1*Se2+covp12
Se1and3 <- Se1*Se3+covp13
Se2and3 <- Se2*Se3+covp23
Sp1and2 <- Sp1+Sp2-Sp1*Sp2-covn12
Sp1and3 <- Sp1+Sp3-Sp1*Sp3-covn13
Sp2and3 <- Sp2+Sp3-Sp2*Sp3-covn23
Sp1or2 <- Sp1*Sp2+covn12
Sp1or3 <- Sp1*Sp3+covn13
Sp2or3 <- Sp2*Sp3+covn23
Se1or2 <- 1-((1-Se1)*(1-Se2)+covp12)
Se1or3 <- 1-((1-Se1)*(1-Se3)+covp13)
Se2or3 <- 1-((1-Se2)*(1-Se3)+covp23)

## test LR values
LRp1 <- Se1/(1-Sp1)
LRn1 <- (1-Se1)/Sp1
LRp2 <- Se2/(1-Sp2)
LRn2 <- (1-Se2)/Sp2
LRp3 <- Se3/(1-Sp3)
```

```
LRn3 <- (1-Se3)/Sp3
LRn1and2 <- (1-Se1and2)/Sp1and2
LRp1or2 <- Se1or2/(1-Sp1or2)
LRn1or2 <- (1-Se1or2)/Sp1or2
LRp1and2 <- Se1and2/(1-Sp1and2)
LRn1and3 <- (1-Se1and3)/Sp1and3
LRp1or3 <- Se1or3/(1-Sp1or3)
LRn1or3 <- (1-Se1or3)/Sp1or3
LRp1and3 <- Se1and3/(1-Sp1and3)
LRn2and3 <- (1-Se2and3)/Sp2and3
LRp2or3 <- Se2or3/(1-Sp2or3)
LRn2or3 <- (1-Se2or3)/Sp2or3
LRp2and3 <- Se2and3/(1-Sp2and3)

## NPV and PPV by prevalence
for (i in 1:length(prev[])) {
        # Individual tests
                NPV1[i] <- Sp1*(1-prev[i])/((1-Se1)*prev[i]+Sp1*(1-prev[i]))
                PPV1[i] <- Se1*prev[i]/(Se1*prev[i]+(1-Sp1)*(1-prev[i]))
                NPV2[i] <- Sp2*(1-prev[i])/((1-Se2)*prev[i]+Sp2*(1-prev[i]))
                PPV2[i] <- Se2*prev[i]/(Se2*prev[i]+(1-Sp2)*(1-prev[i]))
                NPV3[i] <- Sp3*(1-prev[i])/((1-Se3)*prev[i]+Sp3*(1-prev[i]))
                PPV3[i] <- Se3*prev[i]/(Se3*prev[i]+(1-Sp3)*(1-prev[i]))

                # OR-rule
                NPV1or2[i] <- Sp1or2*(1-prev[i])/((1-Se1or2)*prev[i]+Sp1or2*(1-prev[i]))
                PPV1or2[i] <- Se1or2*prev[i]/(Se1or2*prev[i]+(1-Sp1or2)*(1-prev[i]))
                NPV1or3[i] <- Sp1or3*(1-prev[i])/((1-Se1or3)*prev[i]+Sp1or3*(1-prev[i]))
                PPV1or3[i] <- Se1or3*prev[i]/(Se1or3*prev[i]+(1-Sp1or3)*(1-prev[i]))
                NPV2or3[i] <- Sp2or3*(1-prev[i])/((1-Se2or3)*prev[i]+Sp2or3*(1-prev[i]))
                PPV2or3[i] <- Se2or3*prev[i]/(Se2or3*prev[i]+(1-Sp2or3)*(1-prev[i]))

                # AND-rule
```

```
                NPV1and2[i] <- Sp1and2*(1-prev[i])/((1-Se1and2)*prev[i]+Sp1and2*(1-
prev[i]))
                PPV1and2[i] <- Se1and2*prev[i]/(Se1and2*prev[i]+(1-Sp1and2)*(1-prev[i]))
                NPV1and3[i] <- Sp1and3*(1-prev[i])/((1-Se1and3)*prev[i]+Sp1and3*(1-
prev[i]))
                PPV1and3[i] <- Se1and3*prev[i]/(Se1and3*prev[i]+(1-Sp1and3)*(1-prev[i]))
                NPV2and3[i] <- Sp2and3*(1-prev[i])/((1-Se2and3)*prev[i]+Sp2and3*(1-
prev[i]))
                PPV2and3[i] <- Se2and3*prev[i]/(Se2and3*prev[i]+(1-Sp2and3)*(1-prev[i]))
        }
    }
```