A bacteriological disease investigation in a captive breeding colony of Lord Howe Island Stick insects

*(Dryococelus australis)*

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Submitted in fulfilment of the requirements of the degree in

Master of Veterinary Science.

April 2017

Faculty of Veterinary and Agricultural Sciences
The University of Melbourne
ABSTRACT

The Lord Howe Island stick insect (*Dryococelus australis*) is critically endangered in the wild. Once driven to near-extinction, the species now exists within just a few captive breeding programs that have been established to conserve the species, to increase population numbers through breeding, and to support the eventual reintroduction of the species into the wild. In the current study, the bacteria *Serratia marcescens* was investigated as a possible cause of mortality in a captive breeding colony of *D. australis* housed at Melbourne Zoo. *S. marcescens* was isolated from both dead insects and from the environment. The prevalence of the bacteria in dead insects was estimated, and the gross pathological and histopathological changes in infected insects were characterised. Strain types of *S. marcescens* were characterised using pulsed-field gel electrophoresis, and the strain type A1 was implicated as the most likely outbreak strain associated with insect deaths. Epidemiological risk factors for mortality were also investigated, and a novel PCR test was developed to facilitate rapid detection and identification of *S. marcescens*. The overall findings of this research suggest that *S. marcescens* is a potential opportunistic pathogen of *D. australis*, and is associated with haemocoelomitis and death. However, high virulence was not demonstrated in infected insects under experimental conditions. Recommendations for the future management of *D. australis* and the future reintroduction of the species to the wild are discussed in light of the findings from this research.
DECLARATION

This is to certify that:

(1) The thesis comprises only my original work towards the Master degree except where indicated in the Acknowledgments Section.

(2) Due acknowledgment has been made in the text to all other material used.

(3) The thesis is fewer than 50,000 words in length.
ACKNOWLEDGEMENTS

This thesis was completed as part of the course requirements for the Master of Veterinary Science degree (by research) at the University of Melbourne, in conjunction with the Veterinary Residency clinical program at Melbourne Zoo. The research and clinical components of this two-year combined program were weighted equally.

I would like to take this opportunity to thank the many people who have made this research and clinical residency possible, and have given their support and guidance along the way. To my supervisors, Joanne Allen, Dr. Marc Marenda and Dr. Michael Lynch, thank you for all your endless wisdom, encouragement, and patience through some frustrating and testing times during my research, for steering me in the right directions, tirelessly reviewing my chapters, and pushing me to achieve beyond what I thought was possible. To my research chair, Professor Glenn Browning, thank you for your seemingly constant supply of ideas, optimism and somehow, time. To Dr Simon Firestone and Professor Mark Stevenson, thank you for lending me some much needed statistical and epidemiological guidance.

To the Melbourne Zoo veterinarians, veterinary nurses, veterinary keepers and veterinary support officer, I am forever grateful for the opportunity that I had to work with and learn from each of you. If I have somehow gained just a hint of Dr. Helen McCracken’s wisdom and dedication to science, Dr. Kate Bodley’s incredible work ethic and meticulousness, Dr. Sarah Frith’s enthusiasm and fearlessness, and Dr. Michael Lynch’s professionalism and people skills, I will forever be a better veterinarian for it. To all the veterinary support staff, thank you for all your support and for allowing me to occupy much of the veterinary department post-mortem room, hallway, fridges and freezers with my vast collection of research samples.

To the Melbourne Zoo invertebrate keepers, especially Rohan Cleave and Kate Pierce, thank you for sharing both your workspace and your wealth of knowledge in a species so close to your hearts. Special thanks to Rohan for your magnificent efforts in collating data for the epidemiological investigation in this study. And to all the other Melbourne Zoo animal keepers, for having trust in me to care for the zoo’s many wonderful creatures. Thank you to
Zoos Victoria for making this research project possible, for funding my research stipend, and allowing me to be a part of the zoo family; and to both Zoos Victoria and Michael Magrath for funding the histopathological testing of insects in this study.

To the pathologists at Gribbles Pathology, especially to Dr. Christine Bayley, thank you for all your pathology expertise, hard work in performing stick insect histopathology, and your continued enthusiasm throughout what has been a very steep learning curve in phasmid pathology.

To the many other staff and students at the Asia Pacific Centre for Animal Health at the University of Melbourne who have been my sounding board for research ideas and troubleshooting in the laboratory, as well as great company over coffee, very late lunches, and extremely late laboratory sessions, thank you also.

Finally, to my friends and family, thanks for your patience and encouragement through many long, challenging days and absences from your lives.

I cannot thank you all nearly enough for your contributions and support on this journey!
# TABLE OF CONTENTS

Abstract ......................................................................................................................................... i
Declaration ................................................................................................................................... ii
Acknowledgements ..................................................................................................................... iii
List of figures ............................................................................................................................... ix
List of tables ................................................................................................................................ xi
List of abbreviations ................................................................................................................... xiii

## CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW .......................................................... 1

1.1. INTRODUCTION ..................................................................................................................... 1
1.2 THE LORD HOWE ISLAND STICK INSECT ................................................................................. 1
  1.2.1 History of *D. australis* ....................................................................................................... 1
  1.2.2 Conservation objectives and challenges ......................................................................... 3
  1.2.3 Life history and biology of *D. australis* ............................................................................ 3
  1.2.4 Climate conditions on Lord Howe Island......................................................................... 4
1.3 BACTERIOLOGICAL DISEASE INVESTIGATION ......................................................................... 4
  1.3.1 Historical disease in the colony ....................................................................................... 4
  1.3.2 Preliminary disease investigation .................................................................................... 5
  1.3.3 *Serratia marcescens* ........................................................................................................ 5
1.4 RESEARCH AIMS AND APPROACHES .................................................................................... 10
  1.4.1 Overall aims ................................................................................................................... 10
  1.4.2 Specific research aims and approaches ........................................................................ 10

## CHAPTER 2. GENERAL METHODOLOGY AND MATERIALS ....................................................... 12

2.1 STUDY SUBJECTS AND LOCATION ........................................................................................ 12
2.2 HUSBANDRY OF THE *D. AUSTRALIS* COLONY ..................................................................... 12
2.3 EUTHANASIA ........................................................................................................................ 13
2.4 GENERAL BACTERIOLOGY ..................................................................................................... 14
  2.4.1 Culture media ................................................................................................................ 14
  2.4.2 Isolation of *S. marcescens* ............................................................................................. 14
2.5 PULSED-FIELD GEL ELECTROPHORESIS (PFGE) ..................................................................... 15
  2.5.1 Background .................................................................................................................... 15
  2.5.2 Preparation of PFGE gel blocks and cell lysis ................................................................. 15
  2.5.3 Restriction endonuclease digestion ................................................................................. 16
  2.5.4 Electrophoresis and imaging ......................................................................................... 16
2.5.5 Band analysis and dendrogram construction ............................................................... 17
2.6 DNA EXTRACTION ........................................................................................................... 18
2.7 POLYMERASE CHAIN REACTION (PCR) ........................................................................ 18
  2.7.1 Primer design .............................................................................................................. 18
  2.7.2 PCR protocol ............................................................................................................... 19
  2.7.3 Conventional gel electrophoresis and imaging ........................................................ 20
2.8 DNA SEQUENCING ........................................................................................................ 20

CHAPTER 3. POST-MORTEM EXAMINATION AND BACTERIOLOGY ......................... 22

  3.1 INTRODUCTION .............................................................................................................. 22
  3.2 MATERIALS AND METHODS ......................................................................................... 22
    3.2.1 Specimen selection and data collection .................................................................... 22
    3.2.2 Specimen, sample and isolate naming ..................................................................... 23
    3.2.3 Post-mortem examination and sample collection .................................................... 24
    3.2.4 Bacteriological culture and identification ............................................................... 26
  3.3 RESULTS ....................................................................................................................... 29
    3.3.1 Overview of insects sampled ..................................................................................... 29
    3.3.2 Gross post-mortem results ....................................................................................... 31
    3.3.3 Bacteriological results ............................................................................................. 33
    3.3.4 Histopathological results ........................................................................................ 35
  3.4 DISCUSSION .................................................................................................................. 39
  3.5 CONCLUSION ................................................................................................................ 41

CHAPTER 4. EPIDEMIOLOGICAL INVESTIGATION OF MORTALITY RATES IN THE STICK INSECT COLONY ......................................................................................... 43

  4.1 INTRODUCTION .............................................................................................................. 43
  4.2 METHODS ....................................................................................................................... 43
    4.2.1 Data collection ......................................................................................................... 44
    4.2.2 Calculations and assumptions .................................................................................. 45
    4.2.3 Data analysis .......................................................................................................... 47
  4.3 RESULTS ....................................................................................................................... 49
    4.3.1 Distribution of initial count and mortality rate data .................................................. 49
    4.3.2 Effects of sex, glasshouse and month ...................................................................... 51
    4.3.3 The effect of year ...................................................................................................... 54
    4.3.4 The effect of population size .................................................................................... 56
    4.3.5 The effect of local ambient temperature ................................................................. 58
4.3.6 Comparison of the post-mortem investigation period to other months............... 60
4.4 DISCUSSION...................................................................................................................... 61
4.5 CONCLUSION.................................................................................................................... 64

CHAPTER 5. PFGE TYPING OF Serratia Marcescens Isolated from Stick Insects at Post-Mortem.......................................................................................................................... 65

5.1 INTRODUCTION.................................................................................................................. 65
5.2 MATERIALS AND METHODS .......................................................................................... 65
  5.2.1 Selection of isolates and modifications to methodology .............................................. 66
  5.2.2 Dendrogram construction and type analysis ................................................................. 69
  5.2.3 Statistical analysis....................................................................................................... 69
5.3 RESULTS............................................................................................................................. 70
5.4 DISCUSSION....................................................................................................................... 75
5.5 CONCLUSION...................................................................................................................... 77

CHAPTER 6. ENVIRONMENTAL SCREENING .................................................................. 78

6.1 INTRODUCTION.................................................................................................................. 78
6.2 METHODS........................................................................................................................... 78
  6.2.1 Sample collection ......................................................................................................... 78
  6.2.2 Sample processing and plating .................................................................................... 80
  6.2.3 Subculture and colony testing....................................................................................... 81
  6.2.4 PFGE analysis............................................................................................................. 81
6.3 RESULTS............................................................................................................................. 82
  6.3.1 Culture results ............................................................................................................. 82
  6.3.2 PFGE results................................................................................................................. 84
6.4 DISCUSSION....................................................................................................................... 86
6.5 CONCLUSION...................................................................................................................... 89

CHAPTER 7. EXPERIMENTAL INFECTION TRIAL ............................................................ 91

7.1 INTRODUCTION.................................................................................................................. 91
7.2 METHODS........................................................................................................................... 91
  7.2.1 Study strain .................................................................................................................. 91
  7.2.2 Study location and duration ....................................................................................... 92
  7.2.3 Study subjects............................................................................................................. 92
  7.2.4 Study design ............................................................................................................... 92
  7.2.5 Husbandry ................................................................................................................ 94
LIST OF FIGURES

Figure 3.1. Barplots of the number of insects represented by each glasshouse, sex, age class, location found, manner of death, body condition, haemolymph volume and degree of autolysis, of the 80 insects examined post mortem ................................................................. 30

Figure 3.2. Photographs of two adult Dryococelus australis at post-mortem examination, positioned in dorsal recumbency with the ventral exoskeleton reflected cranially to reveal the haemocoel .................................................................................................................. 33

Figure 4.1. Histograms of crude ‘initial count’ data for the population of stick insects .......... 50

Figure 4.2. Histograms of crude mortality rate (deaths per 100 insects per week) ............... 50

Figure 4.3. Boxplot of mortality rate (deaths/100 insects/week) for each sex .................... 52

Figure 4.4. Boxplot of mortality rate (deaths/100 insects/week) for each glasshouse .......... 52

Figure 4.5. Boxplot of mortality rate (deaths/100 insects/week) for each month ............... 53

Figure 4.6. Boxplot of mortality rate (deaths/100 insects/week) for each year .................. 55

Figure 4.7. Scatterplots of initial counts against mortality rate (deaths/100 insects/week) for the overall population and for each glasshouse .................................................................................................................. 57

Figure 4.8. Boxplot of initial counts (per glasshouse) against mortality rate .................... 57

Figure 4.9. Scatterplots of mortality rate against monthly mean maximum daily temperature (°C) for time intervals from zero (same month) to five months prior to mortality ........................................ 59

Figure 4.10. Boxplot of mortality rate (deaths/100 insects/week) during the three-month post-mortem investigation period (period B) compared to all other months between 2010 and 2014 (period A) ................................................................. 60

Figure 5.1. Results of the initial PFGE analysis for genomic DNA extracted from Serratia marcescens isolated from haemolymph at post-mortem examination ................................. 70
Figure 5.2. Dendrogram based on the initial PFGE analysis that featured *Serratia marcescens* isolated from haemolymph at post-mortem examination ............................................................ 71

Figure 5.3. Results of the second PFGE gel for genomic DNA extracted from *Serratia marcescens* isolated from haemolymph at post-mortem examination ............................................................ 72

Figure 5.4. Dendrogram based on the second PFGE analysis that featured *Serratia marcescens* isolated from haemolymph at post-mortem ................................................................................. 73

Figure 6.1. Dendrogram comparing the PFGE analysis results between *Serratia marcescens* isolated from the environmental screening investigation to pure-culture haemolymph isolates from the post-mortem investigation ............................................................ 85

Figure 7.1. Diagrams of the set-up of (a) each mesh enclosure and (b) the glasshouse during the experimental infection trial .......................................................................................................... 93

Figure 7.2. Timeline of pre-infection screening, treatment, post-infection frass monitoring and euthanasia in the experimental infection trial .............................................................................. 95

Figure 7.3. PCR results for the inoculating strain of *Serratia marcescens*, AM923, the isolate cultured from the haemolymph of insect E8-1, and DNA extracted from the haemolymph of insect E8-1 .................................................................................................................................... 103

Figure 7.4. PCR results for DNA extracted from the haemolymph of each of the 40 insects in the experimental infection trial, a colony of inoculating strain AM923, and a colony of isolate E8-1 ...................................................................................................................................................... 104

Figure 7.5. PFGE results for *Serratia marcescens* isolates cultured from pre-infection water samples, post-infection frass samples, the control broth contaminant, and haemolymph from insect E8-1, compared to the inoculating strain, AM923 ........................................................................................................ 108

Figure 7.6. Dendrogram comparing PFGE results for *Serratia marcescens* isolates from the experimental infection trial to pure-culture post-mortem haemolymph isolates, environmental screening isolates and control isolates VW347 and VW348 from the preliminary disease investigation ................................................................................................................. 109
LIST OF TABLES

Table 2.1. Reagent volumes used for each 20 µL Big Dye Terminator sequencing reaction ....... 21

Table 3.1. Criteria for identification of Serratia marcescens, based on the expected biochemical characteristics ................................................................................................................................ 28

Table 3.2. Summary of gross pathological findings from 80 post-mortem examinations .......... 32

Table 3.3. Summary of culture results for 67 haemolymph samples, sorted by bacteria, against culture results that were pure or mixed, and positive or negative for Serratia marcescens .... 34

Table 3.4. Bacteriological culture results compared against post-mortem examination results.. 35

Table 3.5. Summary of histopathological results for eight stick insects from which Serratia marcescens was isolated from haemolymph in pure culture ........................................................ 37

Table 4.1. Summary of data distribution for initial count and mortality rate (deaths/100 insects/week), for each sex and each glasshouse ................................................................. 51

Table 4.2. Outputs of multivariable negative binomial regression to estimate the effects of sex, glasshouse and month on stick insect mortality rates .............................................. 54

Table 4.3. Outputs of multivariable negative binomial regression to estimate the effect of year on mortality rates, corrected for sex, glasshouse and month........................................... 55

Table 4.4. Outputs of multivariable negative binomial regression to estimate the effect of population size (initial count) on mortality rate, corrected for glasshouse and month ............ 58

Table 4.5. Outputs of multivariable negative binomial regression to estimate the effect of monthly mean daily maximum temperature on mortality rate, corrected for glasshouse and sex ......................................................................................................................... 59

Table 5.1. Summary of Serratia marcescens isolates included in the initial PFGE analysis........ 67

Table 5.2. Summary of Serratia marcescens isolates included in the second PFGE analysis ....... 68
Table 6.1. Summary of environmental sample types and sample quantities collected from glasshouse 5 and glasshouse 6 ................................................................................................................................. 79

Table 6.2. Results of DNase testing of selected colonies cultured from frass, water, floor and nest box samples............................................................................................................................ 83

Table 6.3. Seven isolates of *Serratia marcescens* identified from culture of water, nest box, frass and floor samples from glasshouse 5 and glasshouse 6........................................................................... 84

Table 7.1. Summary of PCR results for each treatment group and each enclosure in the experimental infection trial ............................................................................................................................................. 105

Table 7.2. Summary of histopathological results for ten stick insects from the experimental infection trial........................................................................................................................................... 106
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Term</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>+ve</td>
<td>Positive</td>
</tr>
<tr>
<td>-ve</td>
<td>Negative</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µL</td>
<td>Microliter</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
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<tr>
<td>BDT</td>
<td>Big dye terminator</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cfu</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CHX</td>
<td>Cyclohexamide</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>D. australis</td>
<td>Dryococelus australis</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease enzyme</td>
</tr>
<tr>
<td>E</td>
<td>Enclosure</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>Acceleration due to gravity</td>
</tr>
<tr>
<td>GH</td>
<td>Glasshouse</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MAC</td>
<td>MacConkey agar</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>mL</td>
<td>Millilitre</td>
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<tr>
<td>mM</td>
<td>Millimolar</td>
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<tr>
<td>MZ</td>
<td>Melbourne Zoo</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
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<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFG</td>
<td>Pulsed-field gel</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PME</td>
<td>Post-mortem examination</td>
</tr>
<tr>
<td>S. marcescens</td>
<td><em>Serratia marcescens</em></td>
</tr>
<tr>
<td>SBA</td>
<td>Sheep blood agar</td>
</tr>
<tr>
<td>sp.</td>
<td>Species</td>
</tr>
<tr>
<td>spp.</td>
<td>Species (plural)</td>
</tr>
<tr>
<td>Temp.</td>
<td>Temperature</td>
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<tr>
<td>UPGMA</td>
<td>Unweighted Pair Group Method with Arithmetic Mean</td>
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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

The Lord Howe Island stick insect (*Dryococelus australis*) is a critically endangered phasmid (order Phasmida). Once endemic to Lord Howe Island, it was driven to assumed extinction early in the 20th century following the invasion of the European black rat onto the island. Following the rediscovery of a small number of individuals of *D. australis*, a captive breeding colony was established for the species at Melbourne Zoo and has since expanded to consist of over 500 individuals. In recent years, a series of unexpected mortality events and an apparent increase in mortality rate have been observed in this captive population.

1.2 THE LORD HOWE ISLAND STICK INSECT

1.2.1 History of *D. australis*

Once endemic and abundant on Lord Howe Island (31°33’S, 159°05’E), in the South Pacific Ocean, east of New South Wales, Australia, *Dryococelus australis* (Montrouzier, 1855) is now critically endangered in the wild, according to the IUCN Red List of Threatened species (IUCN, 2002). The large, flightless, arboreal insect belongs to the order Phasmida, within the invertebrate family Phasmatidae which includes other stick insect and leaf insect species (Honan, 2008). Other common names for the species include the Lord Howe Island phasmid and the land lobster (IUCN, 2002; Priddel et al., 2003).

Following the introduction of the European black rat (*Rattus rattus*) onto Lord Howe Island by a wrecked trading vessel in 1918, the population of *D. australis* was driven to extinction on the island due to heavy predation by the introduced rat (Priddel et al., 2003). Live insects had not been sighted on Lord Howe Island since the 1920s, and by the early 1930s, *D. australis* was presumed to be globally extinct (Priddel et al., 2003). In the 1960s, the remains of a few recently dead individuals were discovered by rock climbers upon Balls Pyramid, a small rocky, volcanic outcrop, located 25 km off the coast of Lord Howe Island. In the years that followed, multiple attempts were made to survey Balls Pyramid and locate further specimens of *D. australis* on Balls Pyramid, without success (Priddel et al., 2003).
It was not until 2001, 37 years after the first sighting of insect remains, and after seven decades of presumed extinction, that a single, small, living population of *D. australis* was finally rediscovered (Priddel et al., 2003; Carlile et al., 2009). The population, consisting of approximately 20-30 individuals, was found on the side of a cliff on the north-western face of Balls Pyramid (Priddel et al., 2003; Honan, 2007). The entire population survived on only a few shrubs of the endemic tea tree (*Melaleuca howeana*). Currently, the colony on Balls Pyramid remains the only known wild population of *D. australis*, and is thought to consist of fewer than 40 individuals (Honan, 2007).

In 2003, two adult breeding pairs (two females and two males) were recovered from Balls Pyramid and transported to the Australian mainland for the purpose of establishing two captive breeding programs for the species (Honan, 2007, 2008; Carlile et al., 2009). One of those breeding pairs arrived at Melbourne Zoo in Parkville, Victoria, and formed the foundation of the zoo’s conservation program for *D. australis*. Knowledge of the ecology and biology of the species at this time was limited beyond a few brief descriptions published by Montrouzier in 1855, Lea in 1916, and Westwood 1959 (Priddel et al., 2003; Honan, 2007). The second breeding pair was transported to a private invertebrate breeder in Sydney, New South Wales (Honan, 2007).

Apart from a single exchange of four adult males with the New South Wales private collection in an attempt to increase genetic diversity in 2004, no stick insects have been introduced to the colony since the beginning of the program (Honan, 2007). Phenotypic variations, attributed to inbreeding effects, have been noted over the period that this species has been held in captivity. These include a reduction in mean egg size, reduced hatching rates, lower survival rates and physical deformities (Honan, 2007). Genomic studies to evaluate the genetic diversity of the population have yet to be conducted.

Since the breeding program began in 2003, the zoo’s captive population has expanded enormously from the original breeding pair, and has progressed through many generations of insects. At present, the colony consists of between 500-600 individuals (R. Cleave, pers. comm.) and is the largest population of *D. australis* in the world.
1.2.2 Conservation objectives and challenges

The long-term objective of the zoo’s captive breeding program is to increase the number of individuals of *D. australis*, establish a stable captive population of the species, and eventually enable the reintroduction of captive-bred individuals onto Lord Howe Island, to re-establish the wild populations.

In addition to the challenge of successfully establishing a healthy colony of *D. australis* in captivity, a number of other conservation challenges also need to be addressed before successful reintroduction would become possible. At present, the black rat remains widespread on Lord Howe Island, along with the introduced house mouse (*Mus musculus*) posing a major, ongoing risk of predation to any released insects (Carlile et al., 2009). At the current time, political, social and ecological hurdles surrounding the issue of rodent eradication on the island remain unresolved.

1.2.3 Life history and biology of *D. australis*

*D. australis* has an estimated life expectancy of 12-18 months from hatching (Honan, 2008). Females lay either single eggs or batches of approximately 9-10 small, hard-shelled ovoid eggs every 7-10 days, which they deposit either into sedentary substrate, inside nest boxes or on the ground. They begin producing eggs from shortly after reaching adulthood, through until death (Honan, 2008).

*D. australis* is sexually dimorphic, enabling easy identification of the two sexes. Adult females possess a large, distinct ovidepositor sheath on the caudal aspect of the ventral exoskeleton, used to deposit eggs into substrate during laying. Males have enlarged hind limbs that feature femoral spines (Honan, 2008; Carlile et al., 2009). Adult males and females may cluster into social groups or bond as close pairs (Honan, 2008). Reproduction by parthenogenesis has been observed in the absence of males (R. Cleave, pers. comm.).

After 175-245 days of incubation (Honan, 2008), eggs hatch into green-coloured nymphs (juveniles). These nymphs moult through four more instar stages, becoming progressively more brown, before finally moulting into brown-black adults at 201-224 days of age (Honan,
2008). High humidity levels are required to complete each stage of moulting successfully (R. Cleave, pers. comm.).

Nymphs begin with a diurnal lifestyle, foraging high in the foliage throughout the day, and tending to move towards light (R. Cleave, pers. comm.). Adults have a nocturnal lifestyle, emerging to feed, drink and moult during the night (Carlile et al., 2009). All life stages are folivorous, although the plant species comprising the natural diet on Lord Howe Island is unknown (Honan, 2007, 2008). Insects defecate while feeding and resting, producing small, dark, ovoid faecal pellets, termed ‘frass’.

1.2.4 Climate conditions on Lord Howe Island
According to local weather data reported for the Lord Howe Island Aero weather station during the years 1988-2016 (BOM, 2016), the annual mean temperatures recorded on Lord Howe Island range from 17.1°C to 22.1°C, with monthly mean temperatures ranging from 13.5°C (August) to 25.7°C (February). Monthly mean relative humidity levels range from 64% to 71%.

1.3 BACTERIOLOGICAL DISEASE INVESTIGATION

1.3.1 Historical disease in the colony
Case descriptions for a small number of early disease investigations have been reported for the MZ captive population of D. australis (Honan, 2008). Investigative methods included post-mortem examinations, histopathology, radiography, microbiological culture, electron microscopy and toxicological studies. Gross post-mortem changes such as reduction of internal fat stores, reduction in body fluids, and change in gastrointestinal contents were described and used as indicators for disease chronicity and desiccation. While the findings were largely unquantified, and no definitive cause of death was identified, cuticular fractures associated with secondary bacterial invasion and sepsis were suggested as a possible cause of death in five insects (Honan, 2008).
1.3.2 Preliminary disease investigation

In 2013-2014, a number of unusual mortality events and periods of apparent increase in mortality rate were witnessed within the Melbourne Zoo *D. australis* breeding colony (unpublished data). Insects most frequently presented as cases of sudden death, and occasionally presented moribund, in recumbency or with difficulty walking or climbing.

Since the mortalities were first recognised, veterinarians at the Zoo have attempted to determine the cause of deaths through post-mortem examinations, and submitting insect samples to an external commercial laboratory for bacterial culture and histopathological examination. Haemolymph and gut fluid samples were collected for culture, while formalin-fixed whole body tissues were submitted for histopathology. From these early investigations, the bacteria *Serratia marcescens*, was frequently isolated in either pure or mixed culture, while *Pseudomonas* sp. was occasionally isolated (unpublished data). Gross pathology included dark, intrahaemocoelomic plaque lesions in some specimens. Histopathology demonstrated melanin-rich haemocyte lesions, likened to granulomas in mammalian pathology, within haemocoelomic tissues. Some of these lesions contained encapsulated Gram negative bacilli, consistent with the appearance of both *S. marcescens* and *Pseudomonas* sp. (C. Bayley, unpublished data).

Subsequently, five isolates of *S. marcescens* cultured from insects that died during early 2014 were transferred as live culture to the Asia Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, the University of Melbourne for further characterisation. A preliminary investigation was conducted on the isolates received (J. Allen, unpublished data). The identity of each isolate was confirmed to be *S. marcescens* using commercial bacteriological test panels (Enterotube™ II, BD BBL™). The DNA of each isolate was compared using pulsed-field gel electrophoresis, which showed similar DNA fingerprints in four out of five isolates.

1.3.3 *Serratia marcescens*

*S. marcescens* is a Gram negative bacillus of the family Enterobacteriaceae (Hejazi and Falkiner, 1997). It has been implicated in a wide variety of nosocomial infections in humans, including urinary tract, respiratory and wound infections, meningitis, endocarditis and
Septicaemia (Hejazi and Falkiner, 1997). In humans, infections are usually opportunistic and seen mostly in immunocompromised patients in hospital, including neonates, while infections in healthy individuals are uncommon (Miranda et al., 1996; Grimont and Grimont, 2006). The route of entry may be oral, or via a wound, injection site, or catheter site.

*S. marcescens* has the ability to survive in a wide range of environmental conditions and utilise a wide range of nutrients (Grimont and Grimont, 2006). It has been described as saprophytic (Hejazi and Falkiner, 1997) and has a predilection for moist environments (Yu, 1979; Miranda et al., 1996; Passaro et al., 1997; Jones et al., 2000; Brenner and Farmer, 2005). *S. marcescens* has been isolated from water, soil, plants and animals, including both vertebrate and invertebrate species, and may play a role in the biological cycling of metallic minerals (Grimont and Grimont, 2006). It has been found to survive and grow under extreme conditions, such as within disinfectants, antiseptic solutions and double-distilled water (Hejazi et al., 1997; Grimont and Grimont, 2006).

The biochemical and growth properties of *S. marcescens* facilitate its identification from bacteriological culture. Isolates lacking pigment may appear white, mucoid and opaque or smooth and transparent on nutrient agar. Such colonial morphology does not distinguish *S. marcescens* from other enterobacteria (Grimont and Grimont, 2006). Additionally, *S. marcescens* grows at relatively low temperatures and is tolerant of high salt conditions. Due to its production of DNase enzyme, demonstration of the enzyme's activity on agar media can differentiate colonies of *Serratia* sp. from other enterobacteria with similar morphology. Consequently, various DNase test agar media containing DNA and dye indicators, such as toluidine blue and methyl green, have been developed (Grimont and Grimont, 2006).

*S. marcescens* was classically described to have a characteristic red colour (Hejazi and Falkiner, 1997). Due to its distinct colour, and the original belief that it was non-pathogenic, it was frequently used as a visible biological marker for water quality. The red colour of *S. marcescens* is the result of the production of a red pigment, prodigiosin. Prodigiosin is produced only by certain strains of *S. marcescens*, *S. rubidaea* and *S. plymuthica*, but not other enterobacteria. However, within *S. marcescens*, only biogroups A1 and A2/6 are reported to produce prodigiosin, and a few non-enterobacteria can produce the pigment (Grimont and
Grimont, 2006). Synthesis of prodigiosin by *S. marcescens* requires specific growth conditions, including exposure to oxygen and specific salt concentrations (Hejazi and Falkiner, 1997; Grimont and Grimont, 2006).

Both pigmented (red) and non-pigmented (white) environmental isolates of *S. marcescens* have been described in the literature (Grimont and Grimont, 2006). Isolates associated with clinical disease in humans are rarely pigmented (Hejazi and Falkiner, 1997), suggesting that the prodigiosin pigment is unlikely to be a virulence factor for *S. marcescens*. However, both pigmented and non-pigmented forms of *S. marcescens* have been associated with disease in insects (Grimont and Grimont, 1978; Mohan et al., 2011).

*S. marcescens* also produces lipase, multiple proteases, chitinase, lecithinase, DNase, and the surfactant, serrawettin, which have been suggested to be possible virulence factors for insect infection (Hejazi and Falkiner, 1997; Grimont and Grimont, 2006). Supernatants of *S. marcescens* cultures have been shown to have high levels of proteolytic activity (Flyg et al., 1980). The production of fimbriae, siderophores and cell wall antigens have been implicated as additional virulence factors in human and vertebrate infections (Grimont and Grimont, 2006). Genes of *S. marcescens* that code for haemolysin production, lipopolysaccharide biosynthesis and iron uptake have been identified as virulence factors in the nematode (*Caenorhabditis elegans*) model (Leopold Kurz et al., 2003). Variable cell motility has also been described for this bacteria (Hejazi and Falkiner, 1997).

Haemolytic activity has been demonstrated in strains of *Serratia* spp. from a variety of sources (Ruan and Braun, 1990). *S. marcescens* forms small haemolytic zones on standard blood agar plates, which may be frequently overlooked (Hilger and Braun, 1995). The ability of *S. marcescens* to lyse mammalian erythrocytes has been suggested as an important virulence factor, enabling bacterial cells to acquire iron, an essential nutrient for establishing infection (Poole and Braun, 1988). Two patterns of haemolysis have been recognised in *S. marcescens*: one pattern associated with a cell-bound haemolysin with activity on cells in direct contact, and a second pattern associated with a soluble haemolysin (Grimont and Grimont, 1978, 2006; Braun et al., 1985). Haemolytic activity is most pronounced when the bacterial cells are metabolically active, and is depleted when cells are stored at -18 – 4°C (Braun et al., 1985).
Additionally, the onset and rate of haemolysis is increased when *S. marcescens* cells are grown in iron-limiting media, and depressed in iron-supplemented media (Braun et al., 1985).

The haemolysin of *S. marcescens* was first characterised molecularly by Poole et al. (1988), and was found to be distinct from previously known bacterial haemolysins, including that of *E. coli*. The haemolysin of *S. marcescens* is coded by two chromosomal genes, *shlA* and *shlB* (Poole et al., 1988). The gene *shlB* encodes the 62kDa outer cell membrane protein (ShlB) required to secrete and activate the 165kDa haemolysin protein (ShlA), encoded by 4.8-kb *shlA*, across the cell membrane (Schönherr et al., 1993; Hertle, 2000; Franzon and Santos, 2004). In DNA hybridisation tests, DNA probes for *shlA* and *shlB* were found to be specific for strains within the *Serratia* genus, as no complementary DNA was detected for *E. coli*, *Salmonella typhimurium*, *Proteus mirabilis* and *Proteus vulgaris*, *Enterobacter cloacae*, *Klebsiella* spp., *Aeromonas* sp. and *Yersinia pseudotuberculosis*. The haemolysin genes were thus proposed as a useful marker for *S. marcescens* (Ruan and Braun, 1990). Only *P. mirabilis* has been shown to produce haemolysin proteins (HpmA and HpmB), with some nucleotide sequence and amino acid homology to *Serratia* haemolysin proteins (Uphoff and Welch, 1990; Schönherr et al., 1993).

*Serratia* spp. have been previously isolated from healthy, diseased and live specimens of various insect orders, including: Orthoptera (grasshoppers and crickets), Isopteran (termites), Coleoptera (beetles), Lepidoptera (moths and butterflies), Hymenoptera (bees, ants, wasps and sawflies) and Diptera (flies) (Grimont and Grimont, 1978, 2006). However, the clinical significance of individual *Serratia* species in insect infections is difficult to ascertain from early reports, due to historic taxonomic confusion between the *Serratia* species.

*S. marcescens* has been described as a potential pathogen of insects, capable of causing septicaemia and death following experimental inoculation of the haemocoel in more than 70 insect species (Bucher, 1963), including *Drosophila* flies (Flyg et al., 1980), and has been isolated repeatedly from diseased larvae of the honeybee (*Apis mellifera*) (Grimont and Grimont, 2006). It has also been suggested as a possible insect symbiont, with roles in both nutrition and moulting in the sugar beet root maggot (*Tetanops myopaeformis*). Plants are a potential source of *Serratia* spp. found in the insect digestive tract, however the route of entry
from the gut into the haemocoel are not well understood. Rupture of the gut has been suggested as a possible route of entry (Grimont and Grimont, 2006). In addition to horizontal transmission, vertical transmission of \textit{S. marcescens} via insect eggs has been demonstrated in other insect species, \textit{Heliothis virescens} and \textit{Heliothis zea} (Bell, 1969; Sikorowski and Lawrence, 1998; Inglis and Lawrence, 2001). Both external \textit{egg} surface and internal egg colonisation by the bacteria have been demonstrated, associated with a reduction in \textit{egg} production and hatching rates (Inglis and Lawrence, 2001).

In experimental infections of \textit{Drosophila} flies with a non-pigmented \textit{S. marcescens} strain \textit{Db10}, and mutant strains \textit{Db11} and \textit{Db12}, the bacteria was demonstrated to be pathogenic (Flyg et al., 1980). Bacterial invasion of the haemolymph was detectable in all moribund flies, 5-7 days after administration in food. Following administration, death of 90\% of flies occurred within 9 days and death of all flies occurred within 10 days. The authors suggested that the chronology of this finding was consistent with bacterial penetration of the gut after an initial time delay, before causing septicaemia and death. Injection of the bacteria directly into the haemocoel of flies resulted in rapid death of all flies within 22 hours. The LD_{50} of injected \textit{S. marcescens} was found to be 5-15 cfu/fly (Flyg et al., 1980).

Rapid death following injection of \textit{S. marcescens} into the haemocoel has also been demonstrated in sugarcane borer larvae, \textit{Diatraea saccharalis}, with a 95\% mortality rate after 5 days, but no deaths resulted from feeding a diet contaminated with the bacteria (King et al., 1975). In contrast, a mortality rate of 80\% was demonstrated in cabbage looper larvae (\textit{Trichoplusia ni}) fed \textit{S. marcescens} (Bell, 1969). In the banana weevil, \textit{Cosmopolites sordidus}, exposure to \textit{S. marcescens} via feed was found to kill weevil larvae but not adult weevils (Kaaya et al., 1993).

It appears, therefore, that factors influencing the pathogenicity of \textit{S. marcescens} include the bacteria’s ability to colonise the insect gut, its ability to penetrate the gut and enter the haemocoel, the expression of various virulence factors, host immune defences, and host age (Flyg et al., 1980; Kaaya et al., 1993). A possible toxic effect of extracellular factors secreted by \textit{S. marcescens} on the host immune system has also been suggested (Flyg et al., 1980).
Many different typing methods have been used to compare *S. marcescens* isolates in previous studies of the organism, including antibiograms, pulsed-field gel electrophoresis, protein fingerprinting using SDS-PAGE, phage-typing, random amplified polymorphic DNA PCR (RAPD-PCR) and serotyping techniques (McGeer et al., 1990; Miranda et al., 1996; Hejazi and Falkiner, 1997; Shi et al., 1997).

### 1.4 RESEARCH AIMS AND APPROACHES

#### 1.4.1 Overall aims

The purpose of this study was to investigate the mortalities within the captive population of *D. australis* at Melbourne Zoo. We aimed to investigate *S. marcescens* as a potential bacterial pathogen of *D. australis*, to determine if insect mortalities were associated with infection due to a virulent strain of *S. marcescens*, and to characterise the associated pathological lesions present in dead individuals of *D. australis*. In addition, we aimed to identify risk factors associated with mortality, and develop recommendations for the future management of the *D. australis* breeding program.

#### 1.4.2 Specific research aims and approaches

The specific aims of the research were to:

1) determine if the observed deaths in the *D. australis* breeding colony were associated with a virulent strain of *S. marcescens*;
2) obtain environmental isolates of *S. marcescens* and to identify possible environmental sources of the organism in the breeding facility;
3) molecularly compare and type isolates of *S. marcescens* from insects and from the environment;
4) design a test to enable rapid detection and identification of *S. marcescens* isolates;
5) verify Koch’s postulates and demonstrate the pathogenicity of the most likely outbreak strain of *S. marcescens*;
6) establish a baseline mortality rate for the colony and to compare the mortality rate during the study to the baseline mortality rate;
7) identify potential epidemiological risk factors that could influence mortality rate;
8) develop recommendations for the future management of the colony, with respect to prevention and control of disease; and

9) identify implications and develop recommendations for the future reintroduction of *D. australis* into the wild.
CHAPTER 2. GENERAL METHODOLOGY AND MATERIALS

2.1 STUDY SUBJECTS AND LOCATION

All Lord Howe Island stick insects (*D. australis*) investigated in the current study were housed at the Lord Howe Island stick insect captive breeding facility at Melbourne Zoo (MZ), Parkville, Victoria, Australia. All insects were bred, hatched, and reared at this location. Insects used for the experimental infection trial were treated and housed on-site, and no live insects were removed from the facility during the study.

Post-mortem examinations were conducted at the MZ veterinary department and microbiological culture and testing procedures were undertaken at the Asia Pacific Centre for Animal Health, Faculty of Veterinary and Agricultural Sciences, University of Melbourne, Parkville, Victoria, Australia.

2.2 HUSBANDRY OF THE *D. AUSTRALIS* COLONY

The majority of stick insects in the MZ breeding colony were housed indoors within a series of five simple glass- or plastic- walled buildings of various dimensions, referred to as ‘glasshouses’, and a small number of insects were housed in a separate facility for public display. Nymphs and juvenile insects were reared in groups of up to thirty individuals in separate, rectangular, plastic mesh enclosures, all within a single, dedicated rearing glasshouse. Adult and sub-adult insects were housed together within two other glasshouses in mixed sex and age groups. Within the adult/sub-adult glasshouses, insects ranged freely, without separation into smaller enclosures. Insects were relocated into one of the free-ranging glasshouses once they had moulted into sub-adults. The remaining glasshouses in the facility were either used for research trials, to house additional groups of adult and sub-adult insects, or remained unoccupied at various times during the study.

Within each glasshouse, temperature and humidity were artificially-controlled with the aim of approximating the climate observed at the native home of *D. australis* on Lord Howe Island.
Temperature was maintained within a target range of 22°C to 26°C and humidity within a target range of 70 to 80%. Glasshouse floors were cleaned weekly by hosing with water and disinfected using bleach, periodically.

Feed was provided ad lib as live, potted plants or fresh plant clippings. Plants were replaced as required, usually every second day, depending on the quantity of leaf consumed and the visible condition of each plant upon inspection. The plant species included in the diet were: Tree lucerne or tagasaste (*Chamaecytisus prolifer*), Lord Howe Island tea tree (*Melaleuca howeana*), Morton Bay fig (*Ficus macrophylla*), holly oak (*Quercus ilex*), Baloghia sp. and *Pittosporum* sp. Drinking water was provided in shallow, plastic water dishes, refilled daily from the mains water supply.

Deep trays containing a mixture of sand and peat were provided as substrate for egg-laying. Eggs were collected and placed into reused, plastic film canisters for storage of up to 6 months. To prepare them for hatching, eggs were removed from the canisters and buried a few centimetres deep into sand/peat substrate, to imitate natural egg deposition by the female. Hatching rates were artificially managed with the aim to replace insect losses through mortality and maintain a stable population size.

### 2.3 EUTHANASIA

When required, euthanasia was performed by exposing insects to a high concentration isoflurane anaesthetic gas. Each insect was placed into a glass jar or a zip-lock plastic bag containing a cotton wool ball soaked with 100% isoflurane liquid (*Isothesia*, Henry Schein® North Gate, QLD), to create an isoflurane ‘gas chamber’ as the liquid vaporised. The inhalation method of anaesthesia, using isoflurane in a jar or gas chamber, has been previously described for terrestrial invertebrates (Cooper, 2011; Murray, 2012). Each insect was left in the chamber for a minimum of 30 to 60 minutes and examined intermittently. Insects were deemed to be dead upon cessation of all spontaneous body movement and response to stimulus, with failure to regain movement or responsiveness after removal from the chamber. No euthanised insects showed any physical signs of struggling or evasion during exposure to isoflurane, suggesting that this method of euthanasia was humane.
2.4 GENERAL BACTERIOLOGY

2.4.1 Culture media
Standard Luria broth (LB) (Sezonov et al., 2007) and LB agar plates were prepared in the laboratory and sterilised by autoclaving, then used to prepare cultures of *S. marcescens* during pulsed-field gel electrophoresis experiments (Chapter 5) and the experimental infection trial (Chapter 7). LB agar plates containing cyclohexamide (CHX) (0.125 mg/mL) were prepared for the culture of environmental samples. CHX was included to inhibit the growth of environmental fungi that may have otherwise hindered the detection of *S. marcescens* colonies. The concentration of CHX was adapted from the methods previously described for culturing *Serratia* spp. and other enterobacteria (Dorsey et al., 1970; Downing and Thomson, 2000; Callaghan et al., 2001).

Sheep blood agar (SBA), MacConkey agar with bile salts (MAC), and biochemical test media were obtained from a commercial supplier (Media Preparation Unit, the University of Melbourne, Parkville, VIC). Deoxyribonuclease (DNase) agar containing either methyl green or toluidine blue dye indicators (Farmer et al., 1973; Elder et al., 1977) were obtained from commercial suppliers (Media Preparation Unit; MicroMedia Laboratories, Melbourne, VIC).

2.4.2 Isolation of *S. marcescens*
Single, well-isolated colonies of *S. marcescens* were subcultured on SBA and incubated for 18 hours at 37°C. Colony morphology was visually inspected to confirm pure growth, and confirmed to be *S. marcescens* by biochemical testing (Chapter 3) and/or PCR (described later in this chapter). Colonies were harvested using a sterile swab and suspended in tryptic soy broth containing glycerol (30%) within 1.5 mL cryogenic tubes. Each suspended culture was then stored at -80°C for further investigation.
2.5 PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

2.5.1 Background
Molecular typing methods are used to determine the relatedness of bacterial pathogens in epidemiological studies (Tenover et al., 1995). By characterising and comparing the DNA of different bacterial isolates, molecular typing allows similar and dissimilar isolates to be distinguished, and for the degree of relatedness between isolates to be assessed (Tenover et al., 1995). In epidemiological investigations, molecular typing may allow identification of an outbreak strain, inference of a likely outbreak source, and characterisation of the nature and chronicity of the disease outbreak (Tenover et al., 1995; Shi et al., 1997).

The epidemiology of *S. marcescens* outbreaks have previously been investigated using pulsed-field gel electrophoresis (PFGE) typing, with good reproducibility and discriminatory power (Miranda et al., 1996; Shi et al., 1997; Vigeant et al., 1998; Henry et al., 2001; Ligozzi et al., 2010). The restriction endonuclease enzyme, *Spe*I, was found to be the most suitable enzyme for PFGE studies of *S. marcescens* (Miranda et al., 1996; Shi et al., 1997). PFGE enables discrimination between relatively large fragments of DNA, lending its use to epidemiological studies of bacteria, which possess large DNA chromosomes. Fragments ranging in size between 1kb and 7Mb have been successfully resolved using this technique (Warner and Onderdonk, 2003).

2.5.2 Preparation of PFGE gel blocks and cell lysis
Each isolate of *S. marcescens* was cultured onto sheep blood agar (SBA) from stock cultures and incubated for 18 hours at 37°C. A single colony was used to inoculate LB (5 mL) and then incubated at 37°C for 18 hours. An aliquot (1 mL) of the broth was centrifuged at 13,200 × *g* for 3 minutes to create a concentrated bacterial cell pellet. The cells were washed by removing the supernatant, resuspending the pellet in 500 µL of cell wash buffer (1M NaCl, 10mM Tris-HCl pH 7.6) and pelleted at 13,200 × *g* for 3 minutes. The supernatant was removed and the cells were finally resuspended in 300 µL of wash buffer.

Low melting temperature agarose (SeaPlaque®, Cambrex Bio Science Inc., ME, USA) was prepared at 2% in cell wash buffer, and melted at 95°C for 10 minutes with intermittent
mixing. Both the melted agarose and the buffered bacterial cells were equilibrated to 50°C. Each 300 µL of buffered cell suspension was mixed with 300 µL of the 2% agarose, and the final 1% agarose mixture was pipetted into the 100 µL wells of PFG block moulds (Bio-Rad Laboratories, Hercules, California, USA). The moulds were placed on ice to facilitate gel setting. Once set, 5-6 gel blocks per isolate were released from the mould into 1 mL of EC lysis buffer (6mM Tris-HCl pH 7.6, 100mM EDTA pH 7.5, 1M NaCl, 1% sarcosyl) containing lysozyme (1 mg/mL). The gel blocks were incubated for 18 hours at 37°C to lyse the bacterial cells.

Following cell lysis, the EC lysis buffer was removed and replaced with ESP buffer (0.5M EDTA pH 9.2, 1% sarcosyl) and Proteinase K (1 mg/mL). The gel blocks were incubated at 50°C for 4 days. The ESP buffer was removed and the gel blocks were washed six times with TE buffer (1M Tris-HCl, 0.5M EDTA, pH 8.0), with 30 minutes between each wash.

2.5.3 Restriction endonuclease digestion

Bacterial genomic DNA contained within the gel blocks was digested using the endonuclease restriction enzyme, SpeI (New England BioLabs® Inc., Ipswich, Massachusetts, USA). For each isolate, 3 slices of 1 mm thickness were cut from gel blocks. The slices were placed immediately into a microtube containing of 1 × CutSmart® buffer (New England BioLabs® Inc., Ipswich, Massachusetts, USA) and incubated at room temperature for 30 minutes. After initial incubation, the buffer was removed and replaced with fresh 1 × CutSmart® buffer, and 10 units of SpeI was added. The DNA was incubated at 4°C for 10 minutes, then at 37°C for 18 hours.

The remaining undigested gel blocks were stored in EDTA (0.25M, pH 8.0) at 4°C. When required, repeat PFGE analysis was performed on stored gel blocks. The EDTA storage buffer was removed, and gel blocks were washed a further 3 times in TE buffer, as previously described. New 1 mm slices were cut from the washed gel blocks and the endonuclease restriction digestion step was repeated.

2.5.4 Electrophoresis and imaging

Pulsed-field electrophoresis was performed using CHEF-DR III Pulsed Field Electrophoresis System (Bio-Rad Laboratories, Hercules, California, USA). A 1% agarose gel was prepared using
DNA Grade Agarose (Progen Biosciences, Archerfield, QLD) in 0.5 × TBE buffer (45mM Tris, 45mM boric acid, 1.0mM EDTA pH 8.3). The slices containing digested genomic DNA were loaded into the wells containing TE buffer. A Lambda ladder PFG marker (New England BioLabs® Inc., Ipswich, Massachusetts, USA) was used as a molecular weight marker. The gel was loaded into the chamber and the following conditions were used to separate the DNA fragments: 14°C for 20 hours, at 6.0 V/cm, with a 120° included angle and initial and final switch times of 2.2 and 54.2 seconds, respectively.

At the completion of electrophoresis, the gel was stained in ethidium bromide (0.5 mg/mL) for 10 minutes, then destained in fresh distilled water for approximately 30 minutes. The resultant gel was photographed on a Molecular Imager® ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, California, USA) using Image Lab software (version 3.0; Bio-Rad Laboratories, Hercules, California, USA). Labels were added to gel images using CorelDRAW® Graphics Suite X7 software (version 17.0).

2.5.5 Band analysis and dendrogram construction

To facilitate the identification of similar isolates and isolate typing, dendrograms were constructed based on PGFE band analysis using the software application GelJ (version 2.0) (Heras et al., 2015). Gel images were imported into software and the bands of each sample lane were analysed against the lambda ladder marker lane(s). Each image was digitally rotated for straightness, cropped and optimised for contrast prior to analysis. The software automatically detected a subset of bands, but manual adjustments to lane positions and band positions were required before band analysis was possible. Dendrograms were generated from the analysed images based on the Dice method for similarity, and unweighted pair group method with arithmetic mean (UPGMA) linkage. A 3.0% tolerance level was used to construct each dendrogram.

Whenever isolates were represented in multiple gel images or in multiple lanes, the duplicated lanes were initially included in the analyses as control lanes, but later removed to produce the final dendrograms. The lane with the highest quality band resolution for each isolate was included in the final analysis.
2.6 DNA EXTRACTION

DNA from bacterial culture generated from individual colonies was purified using a High Pure DNA Extraction Kit (Roche Applied Science, Mannheim, Germany), following the manufacturer’s instructions. DNA was eluted in 200 µL of the elution buffer provided in the kit.

For haemolymph swab samples stored at -80°C in RNAlater® stabilisation solution (Ambion®, Austin, Texas, USA), samples were thawed before DNA extraction. Each sample was vortexed for 10 seconds before an aliquot (200 µL) was collected into a new sterile tube. The remaining sample volumes were returned to -80°C. DNA extraction was performed on each sample using a High Pure DNA Extraction Kit (Roche Applied Science, Mannheim, Germany), following the manufacturer’s instructions. At completion of the extraction, DNA was eluted in 200 µL of the provided elution buffer. Extracted DNA was used immediately as template for PCR or stored at -20°C.

2.7 POLYMERASE CHAIN REACTION (PCR)

2.7.1 Primer design

A novel PCR protocol was developed within the current study for the detection of *S. marcescens* DNA, and was used to test samples collected during the experimental infection trial. Oligonucleotide primers were designed using the software package Geneious (version R8.1.2; Kearse et al., 2012), targeting the haemolysin A gene, *shlA*, of *S. marcescens*. In the absence of genomic sequence data for the *S. marcescens* strains present in the current study, the assay was designed to detect *shlA* from the strains of *S. marcescens* described hereafter.

The published whole genome sequences of two epidemiologically-unrelated strains of *S. marcescens* from two phylogenetically distant hosts were used as reference sequences for primer design. Sequences of *shlA* from *S. marcescens* strains Db11 (GenBank ID HG326223.1) isolated from *Drosophila* flies, and a multi-drug resistant human isolate SM39 (GenBank ID AP013063.1) (Flyg and Xanthopoulos, 1983; Iguchi et al., 2014) were compared by pairwise alignment using the Geneious software package. Primers were designed using the ‘primers’
function within Geneious, and only regions of the *shlA* gene that were highly conserved between the two *S. marcescens* strains were considered as potential primer targets. In order to specifically target the *S. marcescens* haemolysin gene, and exclude the *P. mirabilis* haemolysin gene, potential primers were further cross-referenced with the whole genome sequences of two *P. mirabilis* strains: HI4320 (GenBank ID NC_010554) and BB2000 (GenBank ID NC_022000). Only primers without any matching regions in either of the *P. mirabilis* sequences were considered as primer candidates.

The forward primer (ShlA1149F) 5’CTGGTTCTATTCTGGCAAT3’ and the reverse primer (ShlA1621R) 5’CCCAGGAGGGTTTGTCATC3’, targeting a 473 bp segment of the *shlA* gene, were selected. A NCBI GenBank BLASTn search (https://blast.ncbi.nlm.nih.gov) was performed for each potential primer sequence to confirm the specificity of these primers for *S. marcescens*. PCR performed on a strain of *P. mirabilis* (cc19) demonstrated a non-specific amplification product of approximately 900-1000 bp, which was easily distinguished from the smaller 473 bp product for *S. marcescens*.

### 2.7.2 PCR protocol

For colony-PCR, a single bacterial colony for each isolate was selected from agar using a sterile pipette tip. Each colony was suspended in 100 µL of sterile ddH₂O and vortexed for 10 seconds. Each sample was boiled at 95°C for 5 minutes, then allowed to cool to room temperature. An aliquot (2 µL) of boiled colony suspension was included as template in each colony PCR. For extracted DNA-PCR, approximately 50 ng of eluted DNA (2 µL) was included as template in each assay. Genomic DNA extracted from a *S. marcescens* isolate (AM923) and sterile ddH₂O were used as templates for positive and negative template controls, respectively.

Each reaction mixture (25 µL) contained: 2U of *Taq* polymerase (GoTaq® Flexi, Promega, Madison, Wisconsin, USA), 1 × GoTaq® buffer (Promega, Madison, Wisconsin, USA), 1.5 mM Mg²⁺, 0.02 mM (each) deoxynucleoside triphosphates, 0.4 µM (each) primer, and 2 µL template. Thermocycling conditions were as follows: 93°C for 2 minutes (initial denaturation); 35 cycles of 93°C for 15 seconds (denaturation), 50°C for 30 seconds (annealing), 72°C for 45
seconds (extension); 72°C for 10 minutes (final extension); and hold at 10°C. All PCR runs were performed using a T100 Thermocycler (Bio-Rad Laboratories, Hercules, California, USA).

2.7.3 Conventional gel electrophoresis and imaging

Electrophoresis gels were prepared using 2% molecular grade agarose (Bioline, Alexandria, NSW) in 0.5 x TBE buffer with SYBR® Safe DNA gel stain (Invitrogen, Carlsbad, California, USA). Aliquots (3 µL) of each PCR product were loaded into each well. At least one 100 bp HyperLadder™ marker (Bioline, Alexandria, NSW) was included in every gel. Each gel was electrophoresed at 80 V (2.7-3.2 V/cm) for between 1 to 2 hours, then photographed using a Molecular Imager® ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Hercules, California, USA) with a UV transilluminator (Bio-Rad Laboratories, Hercules, California, USA) and Image Lab software (version 3.0; Bio-Rad Laboratories, Hercules, California, USA). Images were digitally cropped and exported using the Image Lab software. Labels were added to lanes using CorelDRAW® Graphics Suite X7 software (version 17.0).

2.8 DNA SEQUENCING

DNA amplified by the shlA PCR was purified, sequenced and compared to the expected gene sequence. DNA was purified from PCR products using the UltraClean™ GelSpin DNA extraction kit (Mo Bio Laboratories Inc., Solana Beach, California, USA), according to the manufacturer’s instructions. Purified DNA was eluted in 20 µL of the provided elution buffer. For each sample, 50 ng of purified DNA was used as a template for Big Dye Terminator (BDT) reaction (version 3.1). Sequencing reactions were performed in both directions for each PCR product. A forward reaction was performed using the forward PCR primer (ShlA1149F) and a reverse reaction was performed using the reverse PCR primer (ShlA1621R).

The reagent concentrations used for each BDT reaction (20 µL) are shown in Table 2.1. The volume of eluted DNA sample calculated to obtain 50 ng of DNA is indicated as V (µL). Thermocycling conditions were as follows: 96°C for 1 minute; 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes; 60°C for 4 minutes; and hold at 15°C. All sequencing reactions were performed using a T100 Thermocycler (Bio-Rad Laboratories, Hercules, California, USA).
Table 2.1. Reagent volumes used for each 20 µL Big Dye Terminator sequencing reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume / 20 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDV 3.1 sequence terminator mix</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>5 × BDT dilution buffer</td>
<td>3.25 µL</td>
</tr>
<tr>
<td>5 µM primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>DNA</td>
<td>V µL</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>(14.25 – V) µL</td>
</tr>
</tbody>
</table>

Products of the sequencing reaction were precipitated using sodium acetate, EDTA and ethanol. The entire reaction volume (20 µL) was transferred into a sterile microtube containing 2 µL of sodium acetate (3.0M, pH 5.2) and 2 µL of EDTA (125mM). Chilled 100% ethanol (50 µL) was added and incubated at -20°C for 1 hour. Following incubation, the mixture was centrifuged at 13,200 x g for 20 minutes and the supernatant was removed. Chilled 70% ethanol (100 µL) was added and the microtube was centrifuged at 13,200 x g for 5 minutes before the supernatant was removed. The microtubes were placed in a heating block at 95°C for 1 minute to dry and remove any residual ethanol from the precipitate.

Precipitated DNA was submitted for sequencing at the Centre for Translational Pathology (Department of Pathology, the University of Melbourne, Parkville, VIC). Electrophorograms were imported into Geneious software for analysis of the sequences. Sequences were examined visually and the ends of each sequence result were trimmed. A pairwise alignment was performed between the forward and reverse sequencing results for each isolate to check for discrepancies. Sequence results were compared between each isolate and to the Db11 (GenBank ID HG326223.1) reference sequence.
CHAPTER 3. POST-MORTEM EXAMINATION AND BACTERIOLOGY

3.1 INTRODUCTION

A post-mortem survey was conducted with the aim of assessing the prevalence of *S. marcescens* in the *D. australis* colony. In addition, we aimed to collect isolates of *S. marcescens* for molecular investigation, and to gain knowledge of the gross and microscopic pathological changes associated with *S. marcescens* infection. For this investigation, all insects that died within a three-month period underwent post-mortem examination and were sampled for bacteriological culture and histopathology. The bacterial isolates collected during this investigation formed the basis for further work to evaluate genetic variation within the *S. marcescens* population, and the development of a PCR screening test. The types and frequencies of gross pathological lesions were characterised, and compared to bacteriological and histopathological findings.

3.2 MATERIALS AND METHODS

3.2.1 Specimen selection and data collection

Insects that presented for post-mortem examination (PME) in the study were either found dead or moribund by keeper staff during the daily servicing of glasshouses. Moribund insects were those judged to be unresponsive to external tactile stimuli and unable to move or stand normally. Insects that were displaying unusual postures such as ventroflexion of the head and body were also included in this moribund category. Moribund insects were often found on the ground or outside of nest boxes during the daytime, when other insects had returned to the nest boxes to rest. Insects that presented moribund were humanely euthanised prior to PME, according to the method described in Chapter 2.

A PME was conducted for all insects that died or were euthanised between 3 August 2014 and 31 October 2014, and the samples collected from those insects formed the basis of the
initial post-mortem bacteriology and histopathology study. A total of 80 insects were examined during this period.

Data recorded for each insect examined were: the date of death, date of PME, glasshouse number, enclosure number (if applicable), sex (male, M; female, F; unknown, U), age class (juvenile, J; sub-adult, S; adult, A), location at discovery and whether the insect died naturally or by euthanasia. In addition, the preservation state of internal tissues, body condition, presence of eggs internally (for females), and the samples collected were noted.

### 3.2.2 Specimen, sample and isolate naming

Each insect was assigned an individual name in order to match the samples, data and subsequent bacterial isolates to each corresponding insect. The naming system also allowed key details for each insect to remain associated with each sample and each bacterial isolate. The following information was included in each insect’s ID: age class (J, S or A), sex (M, F or U), date of death (yymmdd format), glasshouse (G1, G2, G5, G6 or G7) and enclosure (variably named). If two or more insects died on the same day, with the same signalment, in the same location, those insects were distinguished by an additional number suffix (n1, n2 etc.).

For example, an adult, female insect that died on 20 August 2014, and was housed in GH5 was assigned the name ‘AF140820-G5’. If a second adult female had died in GH5 that day, the names would have become ‘AF140820-G5n1’ ‘AF140820-G5n2’. A juvenile, unknown sex insect that died on 30 August 2014 in enclosure ‘E3’ of GH2 was assigned the name ‘JU140830-G2E3’, while a juvenile male that died on 25 September 2014 in enclosure ‘Surplus 1’ of GH2 was named ‘JM140925-G2S1’.

Bacterial isolates were initially named after the insect they were cultured from, but isolate names were later abbreviated to the shortest possible form to uniquely identify that isolate. If multiple bacterial isolates were cultured from one insect, each isolate was assigned an additional suffix (i1, i2 etc.) The month and day of the month were included for all isolates, while the year (2014) was excluded since it was the same for all isolates. For example, the name of an isolate from insect AF140820-G5 was abbreviated to ‘AF820’.
3.2.3 Post-mortem examination and sample collection

Prior to and between each insect PME, dissecting instruments were soaked and scrubbed in instrument disinfectant containing benzalkonium chloride and polyhexamethylene biguanide (F10®SCXD Veterinary Cleaner-Sanitizer, 1:250 dilution, Chemical Essentials Pty Ltd, Mitcham North, VIC), then rinsed thoroughly with clean, running water, and finally soaked in ethanol solution. Each insect was surface-disinfected with 80% ethanol solution (as methylated spirits) and then left to air-dry before commencing dissection. Each insect was examined and sampled individually. Instruments were disinfected with 80% ethanol before each PME and whenever instrument contamination was suspected. New disposable gloves were worn for each insect.

To perform each PME, the insect was placed in dorsal recumbency and incised through the lateral body cuticle that connects the hard dorsal and ventral exoskeleton, using fine scissors and forceps. Extreme care was taken to not penetrate the internal viscera during dissection. Each incision was made in a caudocranial direction and the ventral exoskeleton was reflected to reveal the haemocoel. During the initial three-month PME study, incisions were made bilaterally, along both sutures. This technique was later revised to a unilateral, mid-section, initial incision, in order to prevent any instrument contact with the anal region, and to increase time efficiency when examining large numbers of insects.

A subjective assessment of preservation state was made for each insect at the time of PME based on the appearance, colour and integrity of the internal organs. Insects that appeared severely autolysed were excluded from bacteriological sampling and histopathology. Insects assessed to be in a reasonable to excellent preservation state were sampled for bacteriology and fixed for histopathology. It was not possible to determine the preservation state of the insects from their external appearance in most cases, unless leakage of discoloured body fluids was observed, suggestive of tissue degradation.

Using aseptic technique, the fluid within the haemocoel (haemolymph) was sampled from each insect immediately after the initial opening incision. A sterile culture swab was introduced directly into the haemocoel without contacting the body wall, to collect the haemolymph sample. The collected swab was placed into its receptacle, with or without
transport medium. Culture swabs were stored at 4°C except during transportation and sample processing.

Once the haemolymph swab was collected, the body incision was extended bilaterally and the ventral exoskeleton reflected fully to allow visualisation and dissection of the viscera (Figure 3.2). The body condition, degree of autolysis (preservation state) and the volume of the haemolymph was recorded for each insect. These three categories were subjective assessments, each graded on a three-point scale. Body condition was graded ‘good’, ‘fair’ or ‘poor’ based on the relative amount of fat present within in the haemocoel. Insects with generalised brown discolouration and tissue liquefaction were recorded as ‘autolysed’, while insects with good organ integrity and minimal tissue discolouration (other than focal lesions) were recorded as ‘fresh’. The degree of autolysis was further differentiated subjectively into ‘mild’ and ‘severe’ grades. Haemolymph volume was graded from ‘high’, ‘moderate’ or ‘low’. ‘High’ haemolymph insects were those in which haemolymph immediately ejected from the body upon initial incision. Insects graded with ‘moderate’ haemolymph contained sufficient internal fluid for internal organs to appear moist and glistening, while ‘low’ haemolymph insects were those with minimal free fluid and tacky internal organ surfaces, suggestive of hypovolaemia or dehydration.

The digestive tract was examined, including the surface appearance, the presence of digesta and the appearance of the gut contents. Prior to incising the digestive tract and examining the internal contents, gentle digital pressure was applied externally to the foregut, to determine patency of the gut, detected by the movement of digesta along the digestive tract. The Malpighian tubules, respiratory tubules, and reproductive organs were examined. The presence of any internal lesions, including suspected granulomatous lesions (haemocyte plaques) or organ discolouration was noted. The presence of any external injuries or eggs were also noted. The gut was incised and the amount and appearance of the gut content, including any discolouration, was recorded. The more severely autolysed insects were examined in limited detail. The limbs were not dissected and examined due to their small size and difficulty of dissection.
Any insects with suspected granulomas, mucoid clots in the gut, or dark and discoloured organs or tubules were considered to have gross lesions. Other interesting findings were recorded but not classified as lesions for the purpose of this investigation.

Once each PME was completed, the entire insect was submerged into 10% neutral buffered formalin to fix the tissues for histopathology. Insects with moderate to severe autolysis were discarded. Fixed tissues were stored at room temperature until the completion of bacteriological testing.

A total of 80 insects were examined at post-mortem. From those, 67 insect haemolymph samples were cultured and 39 insect bodies were fixed in formalin. From the insects that subsequently yielded pure cultures of *S. marcescens* from haemolymph, eight formalin-fixed bodies were submitted for histopathological examination at a commercial pathological laboratory (Gribbles Veterinary Pathology, Clayton, VIC). All histopathological examination and diagnosis was performed by C. Bayley, veterinary pathologist with a special interest in insect pathology. Seven of the eight insects had died naturally, while one insect, AF141020-G1E6, had been euthanised due to severe disease presentation.

The death-sampling interval (days) was calculated for each insect examined, based on the number of days between discovery of each insect’s death or euthanasia, and its PME. Insects were examined, on average, 1.4 days after the day of death. Insects sampled for culture were examined, on average, 1.3 days after the day of death, with an overall minimum of 0 days and maximum of 10 days.

### 3.2.4 Bacteriological culture and identification

Haemolymph swab samples were plated directly onto sheep blood agar (SBA), and MacConkey agar (MAC) and incubated aerobically at 37°C for 18 hours. The density of colony growth, and the size, colour, morphology, haemolytic activity, and any evidence swarming activity were noted. Growth and lactose fermentation on MAC were noted. The expected characteristics of *S. marcescens* are summarised in Table 3.1).
Individual bacterial colonies were selected from SBA and subcultured to obtain pure cultures. A Gram stain and an oxidase test were prepared for each isolate. Any colonies that were morphologically consistent with *S. marcescens* (medium to large size, and either pigmented or non-pigmented), and consisting of oxidase-negative, lactase-negative, Gram negative bacilli were selected for further biochemical testing.

Gram negative bacilli that tested positive for oxidase activity were presumptively classified as ‘*Pseudomonas*-like’ if the colonies were dark-grey to green in colour, haemolytic and malodorous on SBA. Gram negative bacilli with colony swarming on SBA and negative lactose fermentation on MAC were presumptively classified as ‘*Proteus* sp.’ Other isolates from the primary culture that tested Gram positive, as cocci, were oxidase positive, or that appeared morphologically distinct from *S. marcescens* were grouped as “other bacteria” and were not identified further.

Biochemical tests were performed for each selected colony, to confirm identification of *S. marcescens*. The test series included: indole, methyl red, Voges-Proskauer, citrate (IMViC), arabinose fermentation, urea hydrolysis (urease) and deoxyribonuclease (DNase) agar. All tests were incubated aerobically at 37°C. The inability of *S. marcescens* to ferment arabinose was used to differentiate it from other species of *Serratia*. Isolates that demonstrated the expected biochemical characteristics of *S. marcescens* (Table 3.1), as described in the literature (Grimont and Grimont, 1978; Farmer et al., 2007), were considered to be positively identified as *S. marcescens*. For suspect isolates with biochemical test results consistent with *S. marcescens* in all but one test (excluding Gram stain, oxidase and lactase), that test was repeated in case of a spurious test result. Haemolysis was not consistently documented and was not used as a determining characteristic.
Table 3.1. Criteria for identification of *Serratia marcescens*, based on the expected biochemical characteristics (Grimont and Grimont, 1978; Farmer et al., 2007).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Expected test result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony morphology</td>
<td>Mucoid</td>
</tr>
<tr>
<td>Colony colour</td>
<td>Non-pigmented (white to clear) or pigmented (red)</td>
</tr>
<tr>
<td>Gram staining</td>
<td>Gram negative bacilli</td>
</tr>
<tr>
<td>Lactose fermentation</td>
<td>Negative</td>
</tr>
<tr>
<td>Red pigment</td>
<td>Variable</td>
</tr>
<tr>
<td>Oxidase</td>
<td>Negative</td>
</tr>
<tr>
<td>Methyl red</td>
<td>Negative</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>Positive</td>
</tr>
<tr>
<td>Indole</td>
<td>Negative</td>
</tr>
<tr>
<td>Citrate</td>
<td>Positive</td>
</tr>
<tr>
<td>Arabinose fermentation</td>
<td>Negative</td>
</tr>
<tr>
<td>Urease (urea hydrolysis)</td>
<td>Variable, low</td>
</tr>
<tr>
<td>Deoxyribonuclease (DNase) activity</td>
<td>Positive</td>
</tr>
</tbody>
</table>

For each insect sampled, the presence or absence of *S. marcescens*, ‘*Pseudomonas*-like’ bacteria, *Proteus* sp., or ‘other bacteria’ was recorded. Samples that yielded no visible bacterial growth were recorded as negative cultures. The total number of isolate types detected for each sample was also recorded.

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of PME gross findings for *S. marcescens*-positive haemolymph cultures were calculated. To test the relationship between culture and PME results, a chi-squared test for independence was performed between the two result types, using the statistical software, R (version 2.14.2). Positive and negative culture results for *S. marcescens* were compared against the presence or absence of gross lesions.
All isolates identified as *S. marcescens* using conventional bacteriology and biochemical testing were subcultured on SBA, then stored as stock culture according to the methods described in Chapter 2.

### 3.3 RESULTS

#### 3.3.1 Overview of insects sampled

For the 80 insect PMEs performed in total, the distributions of data for signalment (glasshouse of origin, sex class and age), location of discovery, manner of death, body condition, haemolymph volume, and degree of autolysis are summarised as barplots in Figure 3.1.
Figure 3.1. Barplots of the number of insects represented by each glasshouse, sex, age class, location found, manner of death, body condition, haemolymph volume and degree of autolysis, of the 80 insects examined post mortem. Note the scale of the y-axis varies between plots.

During the PME study, 71 (89%) of the insects that presented had died naturally, while only nine (11%) were euthanised. Glasshouse 5 (GH5) was highly represented compared to the other glasshouses, representing 39% of all insects that were presented for post-mortem examination. This was followed by GH2 (25%), GH6 (19%) and GH1 (11%). Male insects were the more represented sex (48%) compared to female insects (31%). The remaining insects were predominantly juvenile individuals for which sex could not be determined. The adult age
class was most frequently represented (69%), followed by juveniles (21%) and sub-adults (10%). The majority of insects were found either on the floor (39%) of their enclosure or glasshouse, or within a nest box (28%). In total, 4% of dead insects were found attached to the walls of individual mesh enclosures (non-free-ranging insects), 3% were found on feed plants, and one single insect (1%) was found dead in a water dish.

The majority of insects presented in either good body condition with abundant internal fat reserves (30%), or in fair body condition (32%), while insects with little to no fat reserves at the time of death were uncommon (9%). Body condition was not recorded for the remaining 32% of insects. At the time of dissection, 34% of insects were found to have a high volume of haemolymph, while 28% had a moderate volume of haemolymph and 15% of insects had scarce haemolymph. 43% of insects were assessed as “fresh”, without apparent signs of autolysis. Mild and severe autolysis was observed in 21% and 34% of insects respectively.

3.3.2 Gross post-mortem results
For three insects (4%), dark red haemocoelomic plaques consistent with suspect granulomas were observed (Figure 3.2). For 10 insects (13%), a red clot of mucoid material was observed within the foregut or midgut and for 13 insects (16%), a notably large amount of fibrous plant digesta was found within the foregut-midgut region. Physical obstruction of the digestive tract was not identified in any insect. No insects were found to have an empty digestive tract, void of digesta, suggesting that starvation was unlikely to be a contributor to the death of these insects. In seven insects, notable gas was present in the digestive tract, however this may have been an effect of post-mortem change.

Dark, discoloured Malpighian tubes were found in one insect and a dark, discoloured uterus was found in another insect. In autolysed insects, discolouration was more difficult to distinguish from otherwise autolysed tissues. Three insects had limb injuries with missing leg segments, and one insect had died in the process of its final moult. One further insect had a visible penetrating wound in the lateral cuticle associated with a history of ante-mortem needle puncture as part of another research project, which had occurred four days prior to the insect’s moribund presentation and euthanasia. Eggs were found within the oviducts in 11 of the 25 female insects examined. In ten out of those 11 females containing eggs (91%),
the egg count exceeded 10 eggs. It appeared that females frequently remained fecund until death.

A summary of the gross post-mortem findings is presented in Table 3.2. Overall, gross lesions suggestive of infectious disease aetiology were detected in 15 out of the 80 insects (19%), including insects with either suspected granulomas, mucoid clots in the gut, or dark, discoloured organs. Photographs showing examples of a male, adult insect with no visible gross pathology, and female, adult insect with a large haemocoelomic granuloma, confirmed by histopathology, are shown in Figure 3.2.

**Table 3.2.** Summary of gross pathological findings from 80 post-mortem examinations. The percentage frequency of each finding has been rounded to the nearest 1%.

<table>
<thead>
<tr>
<th>Gross lesion type</th>
<th>Number of insects affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any lesion</td>
<td>15/80 (19%)</td>
</tr>
<tr>
<td>Suspected haemocoelomic granuloma</td>
<td>3/80 (4%)</td>
</tr>
<tr>
<td>Red, mucoid clot in foregut or midgut</td>
<td>10/80 (13%)</td>
</tr>
<tr>
<td>Dark, discoloured Malpighian tubes</td>
<td>1/80 (1%)</td>
</tr>
<tr>
<td>Dark, discoloured uterus</td>
<td>1/80 (1%)</td>
</tr>
<tr>
<td>Voluminous plant digesta in foregut</td>
<td>13/80 (16%)</td>
</tr>
<tr>
<td>Gaseous gut</td>
<td>7/80 (9%)</td>
</tr>
<tr>
<td>Empty gut</td>
<td>0/80 (0%)</td>
</tr>
<tr>
<td>Limb or body injuries</td>
<td>4/80 (5%)</td>
</tr>
<tr>
<td>Difficulty moulting</td>
<td>1/80 (1%)</td>
</tr>
<tr>
<td>Eggs present (females)</td>
<td>11/25 (44%)</td>
</tr>
</tbody>
</table>
Figure 3.2. Photographs of two adult *Dryococelus australis* at post-mortem examination, positioned in dorsal recumbency with the ventral exoskeleton reflected cranially to reveal the haemocoel. The male insect (left) showed no visible gross pathological lesions. The female insect (right) was found to have a large, dark haemocoelomic granuloma ventral to midgut (white arrow), and eggs present within the oviducts (black arrow). Grossly normal Malpighian tubules in each insect are indicated (black arrowheads).

3.3.3 Bacteriological results
Haemolymph samples from a total of 67 individual insects were submitted for culture, with *S. marcescens* isolated from 36 (54%) of samples. Twenty-four (36%) of those *S. marcescens* isolates were present in pure culture. Four insects (6%) yielded negative cultures. All isolates of *S. marcescens* demonstrated slow urease enzyme activity with slight positive urease test
results after 24 hours of incubation and strong positive results at 48 hours. All *S. marcescens* isolates were non-pigmented on SBA and MAC. A summary of the results of bacterial culture and identification is presented in Table 3.3. To examine the relationship between the presence of *S. marcescens* and the presence of other bacteria in culture samples, culture results that were positive or negative for *S. marcescens* were cross-tabulated against culture results that were pure or mixed.

**Table 3.3.** Summary of culture results for 67 haemolymph samples, sorted by bacteria, against culture results that were pure or mixed, and positive or negative for *Serratia marcescens*. Percentages shown in the ‘total cultures’ column indicate the proportion of the samples that cultured positive for each bacteria group.

<table>
<thead>
<tr>
<th>Culture result group</th>
<th>Total cultures</th>
<th>Pure cultures</th>
<th>Mixed cultures</th>
<th><em>S. marcescens</em> positive</th>
<th><em>S. marcescens</em> negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total insects cultured</td>
<td>67</td>
<td>39 (58%)</td>
<td>24 (36%)</td>
<td>36 (54%)</td>
<td>31 (46%)</td>
</tr>
<tr>
<td><em>S. marcescens</em></td>
<td>36 (54%)</td>
<td>24 (36%)</td>
<td>12 (18%)</td>
<td>36 (54%)</td>
<td>–</td>
</tr>
<tr>
<td><em>Proteus</em> sp.</td>
<td>15 (22%)</td>
<td>4 (6%)</td>
<td>10 (15%)</td>
<td>6 (9%)</td>
<td>9 (13%)</td>
</tr>
<tr>
<td><em>Pseudomonas</em>–like*</td>
<td>9 (13%)</td>
<td>4 (6%)</td>
<td>5 (7%)</td>
<td>3 (4%)</td>
<td>6 (9%)</td>
</tr>
<tr>
<td>Other bacteria</td>
<td>24 (36%)</td>
<td>7 (7%)</td>
<td>17 (25%)</td>
<td>7 (10%)</td>
<td>17 (25%)</td>
</tr>
<tr>
<td>Negative culture</td>
<td>4 (6%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

For the 67 insects cultured, the number of insects that were culture-positive for *S. marcescens* was compared to the number of insects that were positive for gross lesions detected at PME, by cross-tabulating the positive and negative results for each test (Table 3.4).
Table 3.4. Bacteriological culture results compared against post-mortem examination results.

<table>
<thead>
<tr>
<th>Post-mortem examination result</th>
<th>S. marcescens culture positive</th>
<th>S. marcescens culture negative</th>
<th>Total insects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion positive</td>
<td>7</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Lesion negative</td>
<td>29</td>
<td>23</td>
<td>52</td>
</tr>
<tr>
<td>Total insects</td>
<td>36</td>
<td>31</td>
<td>67</td>
</tr>
</tbody>
</table>

The sensitivity of PME for the presence of *S. marcescens* was calculated to be 19%, while the specificity was determined to be 74%. The positive predictive value (PPV) of PME was determined to be 47% while the negative predictive value (NPV) was 44%. A chi-squared test for independence performed for PME results against culture results, gave a p-value of 0.55 with 1 degree of freedom. The null hypothesis stating independence between the two variables could not be rejected.

In contrast, for the culture positive insects that were submitted for histopathology, seven out of eight insects (88%) were found to be positive for histopathological lesions. No culture negative insects were submitted for examination.

3.3.4 Histopathological results

The results of histopathological examination of the eight submitted insects are summarised in Table 3.5. For seven out of eight *S. marcescens* positive insects (88%) that were submitted for histopathology, granulomatous haemocyte lesions with bacteria were observed. The eighth insect did not have granulomatous lesions, but was found to have some hyper-cellular changes within the haemocoel. Those changes may have represented an earlier stage of disease progression compared to the other insects. Five insects (63%) had haemocyte granulomas associated with the midgut or the midgut-hindgut junction, and four insects (50%) demonstrated other abnormalities of the haemocoel epithelium. Free intrahaemocoelomic bacteria were confirmed in only two of the insects (25%), and lesions of the head were found in another two insects (25%).

Gross lesions were detected on PME in only one of the seven (14%) insects confirmed to have histopathological lesions, and also in the one insect that was not found to have distinct
haemocoelomic lesions on histopathology. Intrahaemocoelomic bacteria were detectable on histopathology in only two of the insects (25%), despite positive cultures of *S. marcescens* in all eight insects.
Table 3.5. Summary of histopathological results for eight stick insects from which *Serratia marcescens* was isolated from haemolymph in pure culture. Note: categories for which no specific pathologist comments were made are denoted by a dash (–). Pigmentation refers to the presence of microscopic pigment granules within lesions, observed on histopathology.

<table>
<thead>
<tr>
<th>Specimen name / isolate name</th>
<th>Death-sampling interval</th>
<th>Gross lesions</th>
<th>Summary of histopathological findings</th>
<th></th>
<th></th>
<th>Cuticular lesions</th>
<th>Other findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Haemocoelomic granulomas</td>
<td>Intra-lesional bacteria</td>
<td>Gut lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF140828-G5 / AF828</td>
<td>0 days</td>
<td>No</td>
<td>Widespread. Head and body. Pigmented.</td>
<td>Some</td>
<td></td>
<td>Irregular cuticle around head.</td>
<td>Hyperplasia, melanisation of haemocoelomic epithelium.</td>
</tr>
<tr>
<td>AF141006-G5 / AF1006</td>
<td>1 days</td>
<td>No</td>
<td>Widespread in head, limb, body and midgut wall. Pigmented.</td>
<td>Yes</td>
<td>Large haemocyte plaque in midgut.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF141007-G5 / AF1007</td>
<td>0 days</td>
<td>Yes</td>
<td>Widespread in head, limb and hindgut wall.</td>
<td>Often</td>
<td>Large haemocyte plaque at midgut-hindgut junction, extending into haemocoel.</td>
<td>Occasional erosions with embedded mixed bacteria in mouthparts.</td>
<td>Part of oviduct wall effaced by haemocyte plaque with bacteria. Hyperplasia and pigmentation of haemocoelomic epithelium.</td>
</tr>
<tr>
<td>AM141008-G5 / AF1008</td>
<td>1 days</td>
<td>No</td>
<td>Widespread in head, limb and body. Pigmented.</td>
<td>Yes</td>
<td></td>
<td></td>
<td>Localised areas of epithelial melanisation in midgut without plaque formation.</td>
</tr>
<tr>
<td>Strain</td>
<td>Survival</td>
<td>Infection</td>
<td>Lesion Details</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>----------</td>
<td>-----------</td>
<td>--------------------------------------------------------------------------------</td>
<td>--------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF141020-G1E6 / AF1020</td>
<td>0 days</td>
<td>No</td>
<td>Widespread in head, limb and body. Pigmented.</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>Large haemocyte plaque at the midgut-hindgut junction, extending into haemocoel.</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM141025-G6 / SM1025</td>
<td>2 days</td>
<td>Yes</td>
<td>No distinct granulomas.</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>Hyper-cellular areas of haemocoelomic tissue, multifocally.</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The most common abnormality that was detected on PME was the presence of a red, mucoid clot in the foregut or midgut (13% of insects), followed by suspect granulomas (4% of insects). Gross discolouration of either Malpighian tubules or the reproductive tract was rare (2% combined). Those three abnormality types were grouped together and considered to be lesions. A large amount of plant material was present in the foreguts of 16% of insects. While physical obstruction of the digestive tract was not confirmed in those insects, it remains unclear whether the observation of such quantities of digesta could represent either normal variation, a possible functional outflow obstruction or perhaps simply a continuously good appetite up until the time of death. Another interesting finding was the moderate percentage of females (44%) that were egg-bearing at the time of death, and the high percentage (91%) that were bearing more than 10 eggs.

In light of bacteriological culture results, the detection of gross lesions on PME and the detection of bacteria on histopathology were both relatively poor predictors for the presence of culturable *S. marcescens* in the haemocoel. PME had a low sensitivity and moderate specificity, with both a low PPV and NPV, for *S. marcescens* colonisation, which may reflect the low proportion of insects that had detectable gross lesions, regardless of infection status. The small size of the insects and their associated lesions, the limited available knowledge of the ‘normal’ variation in internal morphology of *D. australis*, and relative inexperience of the author in conducting insect PMEs at the beginning of the study may have also contributed to low rate of detection of gross pathological changes.

The presence of *S. marcescens* was a relatively poor predictor for the presence of detectable gross pathology and, in turn, the detection of gross lesions on PME was a relatively poor predictor of the presence of histopathological lesions. However, a high proportion of culture-positive insects (88%) were found to be positive for histopathological lesions, and hypercellular changes were also detected in the remaining insect. Retrospectively, the further inclusion of culture-negative insects in histopathological testing would have been necessary to assess the specificity of culture results in predicting microscopic lesions.
All *S. marcescens* isolates cultured from insects in this investigation showed slow urease activity, in contrast to the variable to low urease activity of *S. marcescens* described in the literature (Grimont and Grimont, 1978; Farmer et al., 2007). Whether urease plays an important role in the virulence of *S. marcescens* in *D. australis* or facilitates survival in the environment is not known. The genes involved in urease activity could be ideal, unique targets for PCR test design.

Autolysed insects were included in the total count of insects examined in this study. However, the detection of gross pathological lesions in those insects may have been impaired by the degree of autolysis present. For example, severe discolouration and disintegration of tissue structures due to autolysis could have masked subtle pathological changes to tissue and colour. Therefore, the true frequency of gross pathology may be slightly higher than that detected. Additionally, while severely autolysed insects were excluded from culture, the distinction between mild, moderate and severe autolysis was made subjectively based on the appearance of the haemolymph and internal organs, but may not necessarily correlate with tissue integrity. For insects with autolysis affecting the integrity of the gut wall, post-mortem bacterial invasion of the haemocoel may have affected bacteriological culture findings. For these reasons, it may be preferable to exclude insects with any evidence of autolysis in future investigations.

In the initial sampling trials, up to three different sampling techniques were trialled and compared. Haemolymph was collected using both a culture swab and via a needle aspirate from the haemocoel of a hind limb, while gastric contents were also swabbed. Three eggs collected from within the reproductive tracts of female insects were also sampled for culture via needle aspirate.

The yield of fluid obtained by aspirating from the limb was low to undetectable, despite trialling a range of different needle gauges. While culture of gut flora was possible, it was assumed that detection of bacteria within the haemolymph was more likely to represent a clinically significant bacterial colonisation of the haemocoel and sepsis, rather than simply the presence of flora within the gut. Thus, swab collection of haemolymph was found to be the
most consistent, rapid and easy method of obtaining a decent yield of fluid sample for culture, and only this technique was continued past initial testing.

In this current study, almost all PMEs were conducted by the author, and all histopathological examinations were conducted by one pathologist. Further variation in sensitivity and specificity of each test would be expected if further investigators were included in the study, dependent on individual experience, ability to detect changes, and interpretation of the findings.

*S. marcescens* has previously been isolated from the gut contents of other species within the insect order Phasmatodea, including *Extatosoma tiaratum*, *Diapherodes gigantea*, and *Phyllium siccifolium* (Shelomi et al., 2015). However, to the author’s knowledge, this is the first study to describe the isolation of *S. marcescens* from the haemolymph of any phasmid species, and the first to investigate the role of bacteria in haemocoelomic infections in *D. australis*. Histopathological lesions associated with haemocoelomic infections have not been described in other phasmands.

### 3.5 CONCLUSION

The combined findings from PME, bacteriological culture and histopathology suggest that *S. marcescens* is a prevalent organism within the haemolymph of dead stick insects, cultured from more than half of the insects sampled. *S. marcescens* is more frequently associated with histopathological pathology than gross pathology, and the detection of gross lesions on post-mortem examination was a relatively poor predictor of the presence of microscopic lesions.

Post-mortem examination of dead *D. australis* provided an ideal opportunity to collect both samples of haemolymph for bacteriological culture, and tissue samples for histopathology. However, continued investment of time and effort to perform a detailed gross examination for every dead insect in great detail at post-mortem may be unrewarding as a tool for monitoring the infectious disease status of the colony, due to insensitivity of this investigative method. Bacterial culture and histopathology represent more rewarding tools for
investigating intrahaemocoelomic infection in individual insects, and for evaluating the health of the colony, albeit at a higher cost per insect.

Due to the absence of obvious clinical signs, and the low frequency of obvious gross pathology, attributing a definitive cause to death or disease was not possible for the majority of insects examined. The duration of disease prior to death was also unknown. An unknown proportion of the insects that presented for post-mortem examination could have died due to non-infectious causes, or acquired infections secondary to other disease processes. Other infectious aetiologies, such as viral, fungal or protozoal diseases, underlying diseases or immunological dysfunction were not investigated in the current study. Therefore, it is not possible to conclude that all insects from which *S. marcescens* was cultured necessarily died due to primary infection by *S. marcescens*. For these reasons, the findings of this investigation should be interpreted with care.
CHAPTER 4. EPIDEMIOLOGICAL INVESTIGATION OF MORTALITY RATES IN THE STICK INSECT COLONY

4.1 INTRODUCTION

In order to determine the significance and impact of *S. marcescens* infection in a population and to identify epidemiological events associated with increased mortality rates, an understanding of the mortality rate in the population is required. Currently, no baseline mortality rate exists for the captive population of *D. australis* at Melbourne Zoo, and to the author’s knowledge, no mortality rate data has been published for *D. australis* populations elsewhere. The current epidemiological investigation is the first attempt to quantify the rate of mortality in the Melbourne Zoo captive population, with the purpose of providing a reference for objective monitoring and management of future disease outbreaks.

The aims of the current epidemiological investigation were to establish an overall baseline mortality rate, and to identify any risk factors associated with mortality by examining the effects of sex, location, month, year, population size and environmental temperature on mortality rate. In addition, the analyses sought to assess how the insect mortality rate observed during the three-month post-mortem investigation period within the current study (August to October 2014) compared to the ‘historic’ mortality rate over a five year-period (January 2010 to December 2014).

4.2 METHODS

The null hypotheses for this investigation were that: a) no difference exists in mortality rate between males and females, b) no difference exists in mortality rate between glasshouse 5 (GH5) and glasshouse 6 (GH6), c) no differences exist in mortality rate between different months of the year, d) no difference exists in mortality rate due to population size, e) no difference exists in mortality rates during months of low and high local temperatures, and f) no difference exists in the mortality rate during the post-mortem investigation period compared to the rest of the five-year period.
4.2.1 Data collection

Monthly population and mortality data over five years (2010-2014) were collated for two glasshouses, GH5 and GH6, from paper records (R. Cleave, unpublished data). The data represented the population at the start of each month and the number of deaths that occurred during the previous month, for each sex and each glasshouse. Dead insects were counted and removed by keepers each day. Juvenile insects were not included in this investigation, as precise counts of juvenile deaths were not available. Mortality counts included both natural mortalities and euthanised insects, and were likely to include mortalities due to infectious and non-infectious disease processes, and reaching life expectancy (‘old age’). However, for the purpose of establishing an initial baseline mortality rate for the population, differentiation of the various causes of death was not considered critical.

The two glasshouses included in this investigation contained free-ranging, adult and sub-adult insects of both sexes. During the five-year period, GH5 was unpopulated during two time periods: all 12 months of 2010, and another nine months from November 2011 to July 2012 (inclusive). GH6 was populated throughout the five-year period. Non-equal numbers of males and females, and non-equal total population counts for each glasshouse, were represented each month. Intermittently, new insects were moved into each glasshouse as they moulted into sub-adults and adults. Insects included in the data may have also been moved between GH5 and GH6, or to and from the other glasshouses. The exact details (timing, location and reason) for each move were not included in the data.

The two glasshouses were very similar in dimensions and construction, and were situated in close proximity, separated by a small path. The husbandry and layout of furnishings were similar between glasshouses. Feed and drinking water in each glasshouse were provided from the same source.
4.2.2 Calculations and assumptions

Raw data obtained from keeper records were reorganised into columns for each variable (year, month, GH, sex, initial insect count, number of deaths) in Microsoft Excel software (2016 version). The following variables were calculated: ‘days in calendar month’, ‘final count’ and ‘insects moved’. Local monthly temperature data (monthly means of daily maximum temperatures), recorded between August 2009 to December 2014, were obtained from the Australian Bureau of Meteorology (www.bom.org.au) for the Melbourne Regional Office weather station, situated at 37.81S 144.97E (3.2 km away from the stick insect colony).

The ‘final count’, defined as the population size at the end of each month, was considered as the initial population size (‘initial count’) at the start of the following month. Since data for the number of insects added or removed from each glasshouse was not available, the net number of ‘insects moved’ was calculated using the following formula:

\[
\text{insects moved} = \text{final count} - \text{initial count} + \text{number of deaths}
\]

The mortality rate needed to be defined in a way that would incorporate the varying population at risk over time, be relatively straight-forward to interpret and be appreciable on a timescale that would allow timely intervention to occur, should the mortality rate increase unexpectedly. Based on these requirements, the unit ‘deaths per 100 insects per week’ was chosen.

The crude mortality rate (in units of per 100 insects per week) was defined as:

\[
\text{mortality rate} = \frac{\text{deaths}}{\text{animal time at risk}}
\]

To account for the differing number of days in each month, the denominator (animal time at risk) was defined as (Thrusfield, 2005):

\[
\text{denominator} = \left(\text{initial count} - \left(\text{deaths} \div 2\right) + \left(\text{moved} \div 2\right)\right) \times \left(\text{days in month} \div 7\right) \div 100
\]

This approach makes the approximation that all deaths and insect movements occurred in the middle of each month (i.e. animals that died in a given month contributed ½ a month to the denominator which approximates total animal time at risk in that month, thus minus deaths ÷ 2 and plus moved ÷ 2), and that the population at that point in time was equal to the
mean of the initial and final insect counts for that month. This approach aims to provide a reasonable monthly approximation of the mortality rate based on the available data. One death per 100 insect weeks would, for example, equate to one death per 100 insects per week, or one death per one insect per 100 weeks. Thus, the mortality rate (in units of per 100 insects per week) was defined by the overall formula:

\[
\text{mortality rate} = \frac{\text{deaths}}{\text{initial count} - (\text{deaths} \div 2) + (\text{moved} \div 2)} \div (\text{days in month} \div 7) \times 100
\]  

(Mortality rates for months associated with either no final insect count or with an empty glasshouse during the previous month were specially dealt with. Since, final insect counts were not available for December 2014, the last month in the dataset, data for that month was excluded from the analysis. For the months during which a previously unoccupied glasshouse became repopulated (January 2011 and August 2012, both in GH5), the repopulation was assumed to have occurred as a single event in the middle of the month. All insect deaths and movements had to then be assumed to have occurred midway between the repopulation event and the end of the month (¾ of the days through the month), when the population was equal to the mean of the initial and final insect counts. Effectively, this required adjusting the denominator to reflect that animal time at risk was only considered for half of the month (i.e. for Jan 2011 and Aug 2012, in GH5). Therefore, in these months, the mortality rate was defined as:

\[
\text{mortality rate} = \frac{\text{deaths}}{\text{initial count} - (\text{deaths} \div 2) + (\text{moved} \div 2)} \div (\text{days in month} \div 14) \times 100
\]  

To approximate the theoretical expected rate of mortality from insects reaching life expectancy, assuming that all insects had equal life expectancy (of 18 months), and that the frequency of hatching remained constant throughout the year, the expected number of deaths per week was estimated as follows:

\[
\text{theoretical expected mortality rate} = \frac{\text{number of adult insects} \times 7}{365.25} \times \text{deaths per week}
\]

Therefore, for 100 insects, the expected number of deaths would equate to 1.92 deaths per 100 insects per week.
4.2.3 Data analysis

All data analyses were performed using statistical software, R (version 2.14.2; R Core Team, 2016), with the libraries: msm (Jackson, 2012), epiR (Stevenson et al., 2015), MASS (Ripley et al., 2012) and Rmisc (Hope, 2012).

The distribution of the ‘initial count’ (population size) and mortality rate data were examined using histograms and descriptive statistics (n, range, mean, standard deviation and skewness), both overall (crude) and stratifying by sex and glasshouse. Due to the right-sided skew in the mortality rate data, and the ‘count’ nature of the raw mortality data, Poisson and negative binomial regression models (formulated as generalised linear models with the mortality rate defined in terms of the integer number of insect deaths for each month and the denominator as defined previously, included as an ‘offset’ factor) were constructed to estimate the association between mortality rates and each of the following explanatory variables: sex, glasshouse, month, year, temperature and time period.

For each explanatory variable, multivariable regression was undertaken to adjust for confounding with the other variables. The final results were derived from the best fitting model for each analysis, based on comparison of the Akaike Information Criterion (AIC, Akaike, 1998). A negative binomial model was accepted as a better fit for the data than a Poisson regression model if the dispersion factor (1/θ, which generalises the Poisson regression model to account for over-dispersed data) and the lower bound of its 95% confidence interval (CI) were >0. The output coefficients of each regression analysis were exponentiated so they could be expressed as incidence rate ratios (IRRs) with 95% CIs for each variable considered.

The sex, glasshouse and month variables were analysed together using multivariable regression, with males, GH5 and July as the ‘reference’ factors. A median mortality rate was generated for each reference factor. Year, time period, initial count and temperature variables were analysed separately due to the modifications to the dataset that were required to perform analysis. Months were initially plotted and analysed in accordance with the conventional order of months. However, due to the appearance of a sinusoid pattern
observed for mortality rate, months were reordered to follow the ‘financial year’, i.e. beginning in July and ending in June; an approach commonly applied when analysing epidemiological data on arboviruses in the Southern hemisphere (Cutcher et al., 2017).

To analyse the effect of year, each year from 2012 to 2014 was compared to 2011, as the ‘reference’ year. Multivariable negative binomial regression was undertaken, corrected for sex, glasshouse and month. Since GH5 was known to be unpopulated throughout 2010, and due to evidence of unequal mortality rate between the two glasshouses from the previous analysis, 2010 was excluded from the analysis.

To examine the possibility that high stocking density (‘overstocking’) could affect mortality rate, mortality rates were analysed against initial count (population size) data. Data were combined for males and females in each glasshouse to analyse the effect of overall initial counts on overall mortality rates (per glasshouse). Multivariable negative binomial regression was undertaken, adjusting for glasshouse and month, firstly with initial count as a continuous variable and then as a discrete variable. To identify if any critical threshold existed for population size, initial counts were categorised into six discrete classes: 51–100, 101–150, 151–200, 201–250, 251–300, and 301–350 insects. No months were associated with an initial count less than 51 (unoccupied months were excluded). Mortality rates for each class were compared to the ‘reference’ category with the lowest population size (51–100 insects).

To further investigate a possible explanation for the seasonal pattern observed in the previous analysis of mortality rates against months, the effect of local temperatures on mortality rate was also analysed. Mortality rate was compared against the monthly mean of daily maximum temperatures reported for the Melbourne Regional Office weather station. Maximum data was selected due to the apparent summer peak and winter trough in mortality rates observed during the previous analysis for months. To account for both acute effects of temperature (e.g. sudden death) and delayed effects (e.g. slow disease processes), mortality rates were compared to the monthly mean maximum daily temperature (°C) for the corresponding month, and for each month up to 5 months prior. Multivariable negative binomial analysis was undertaken between mortality rates and the temperatures with each time lag, correcting for sex and glasshouse.
To determine how well the post-mortem investigation period in the current study represented the overall incidence of mortality in the population, mortality rates during the post-mortem period were compared to those for the remaining months in the five-year period. The three months of the post-mortem investigation (August-October 2014, ‘period B’) were selected as a subset of the dataset, and compared against all remaining months (56 months, ‘period A’). Multivariable negative binomial analysis was undertaken to compare period A to period B, adjusting for sex, glasshouse and month.

4.3 RESULTS

4.3.1 Distribution of initial count and mortality rate data
The distribution of the initial insect count data (Figure 4.1) suggested a normal to right-skewed data distribution. A similar pattern of initial count distribution was shown for males and females, except for the higher frequency of initial counts exceeding 150 insects for females. The highest frequencies of initial counts occurred in the range of 50–100 insects for each sex. In GH6, the initial count data had a wider range, with more counts exceeding 200 insects compared to GH5. From the distribution of the initial count data, it appeared unlikely that over-representation by either sex would affect the statistical analysis for mortality rate, although higher population sizes would need to be taken into account for GH6 compared to GH5.

The distribution of the mortality rate data (Figure 4.2) was strongly right-skewed, with higher frequencies of low-end mortality rates compared to high-end mortality rates. A similar pattern of distribution was shown for each sex and for each glasshouse.

Descriptive statistics for the initial counts and mortality rates for the overall population, and by sex and glasshouse, are presented in Table 4.1.
Figure 4.1. Histograms of crude ‘initial count’ data for the population of stick insects (top) then by sex (middle) and by glasshouse (bottom).

Figure 4.2. Histograms of crude mortality rate (deaths per 100 insects per week) (top) then by sex (middle) and by glasshouse (bottom).
Table 4.1. Summary of data distribution for initial count and mortality rate (deaths/100 insects/week), for each sex and each glasshouse.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Factor</th>
<th>n</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial count</td>
<td>Either GH</td>
<td>99</td>
<td>153</td>
<td>70, 313</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>99</td>
<td>82</td>
<td>42, 218</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>96</td>
<td>72</td>
<td>30, 137</td>
</tr>
<tr>
<td></td>
<td>GH5</td>
<td>39</td>
<td>147</td>
<td>70, 231</td>
</tr>
<tr>
<td></td>
<td>GH6</td>
<td>60</td>
<td>164</td>
<td>79, 313</td>
</tr>
<tr>
<td>Mortality rate</td>
<td>Either GH</td>
<td>97</td>
<td>2.04</td>
<td>0.08, 8.92</td>
</tr>
<tr>
<td></td>
<td>Females</td>
<td>97</td>
<td>2.02</td>
<td>0, 10.77</td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>94</td>
<td>2.04</td>
<td>0, 11.97</td>
</tr>
<tr>
<td></td>
<td>GH5</td>
<td>38</td>
<td>1.78</td>
<td>0.45, 6.97</td>
</tr>
<tr>
<td></td>
<td>GH6</td>
<td>59</td>
<td>2.34</td>
<td>0.08, 8.92</td>
</tr>
</tbody>
</table>

4.3.2 Effects of sex, glasshouse and month

Mortality rates plotted as boxplots against sex and glasshouse are shown in Figure 4.3. and Figure 4.4 respectively, indicating only slight differences in mortality rates between each factor. Mortality rates plotted as boxplots against each month are shown in Figure 4.5, indicating a peak in mortality rate around the period of summer in southern Australia (December to April), and the trough around the period of winter (June to September), indicating a seasonal pattern of mortality risk. This pattern was also observed when the data was plotted separately for each year (plots not shown).

A summary of the outputs of multivariable negative binomial regression for the association between mortality rate and sex, glasshouse and month is presented in Table 4.2. No statistically significant difference in mortality rate was detected between male and females. Mortality rate was found to be slightly higher in GH6 compared to GH5 (p = 0.025), with moderate statistical significance. The median mortality rate for the reference month (of July) was estimated to be 1.08 deaths/100 insects/week. The months between December and May were associated with statistically significant increases in mortality rate, compared to July.
Figure 4.3. Boxplot of mortality rate (deaths/100 insects/week) for each sex.

Figure 4.4. Boxplot of mortality rate (deaths/100 insects/week) for each glasshouse.
Figure 4.5. Boxplot of mortality rate (deaths/100 insects/week) for each month. Months are ordered according to the financial year (July to June).
Table 4.2. Outputs of multivariable negative binomial regression to estimate the effects of sex, glasshouse and month on stick insect mortality rates.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Levels</th>
<th>IRR</th>
<th>95% CI of IRR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Females</td>
<td>1.00</td>
<td>(reference)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Males</td>
<td>1.03</td>
<td>0.85, 1.25</td>
<td>0.78</td>
</tr>
<tr>
<td>Glasshouse</td>
<td>GH5</td>
<td>1.00</td>
<td>(reference)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GH6</td>
<td>1.27</td>
<td>1.03, 1.55</td>
<td>0.025</td>
</tr>
<tr>
<td>Month</td>
<td>January</td>
<td>2.02</td>
<td>1.24, 3.30</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>February</td>
<td>2.48</td>
<td>1.53, 4.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>March</td>
<td>3.01</td>
<td>1.87, 4.87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>April</td>
<td>2.22</td>
<td>1.37, 3.61</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>May</td>
<td>1.83</td>
<td>1.12, 2.98</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>June</td>
<td>1.14</td>
<td>0.69, 1.90</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>July</td>
<td>1.00</td>
<td>(reference)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>0.70</td>
<td>0.42, 1.19</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>September</td>
<td>1.30</td>
<td>0.79, 2.14</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>October</td>
<td>1.58</td>
<td>0.92, 2.58</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>November</td>
<td>1.50</td>
<td>0.97, 2.47</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>December</td>
<td>1.93</td>
<td>1.14, 3.25</td>
<td>0.014</td>
</tr>
</tbody>
</table>

IRR = incidence rate ratio; AIC = 1163.3, θ (95% CI) = 2.94 (2.11, 3.78), n = 191.

4.3.3 The effect of year

Mortality rates plotted as boxplots against each year (2010 to 2014) are shown in Figure 4.6, which suggests a fluctuation in mortality between alternate years. A summary of the outputs of multivariable negative binomial regression for the association between mortality rate and year, corrected for sex, glasshouse and month, is presented in Table 4.3. Compared to 2011, 2012 and 2014 were associated with significantly decreased mortality rates (p = 0.001). No significant difference was found for 2013. The median mortality rate for 2011 was estimated to be 2.55 deaths/100 insects/week.
Figure 4.6. Boxplot of mortality rate (deaths/100 insects/week) for each year.

Table 4.3. Outputs of multivariable negative binomial regression to estimate the effect of year on mortality rates, corrected for sex, glasshouse and month.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Levels</th>
<th>IRR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>2011</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>0.67 (0.52, 0.85)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>2013</td>
<td>1.14 (0.92, 1.41)</td>
<td>0.229</td>
</tr>
<tr>
<td></td>
<td>2014</td>
<td>0.68 (0.54, 0.86)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

IRR = incidence rate ratio; AIC = 959.33, θ (95% CI) = 7.15 (4.05, 10.25), n = 167.
4.3.4 The effect of population size

Mortality rates plotted as scatterplots against initial count data are shown in Figure 4.7. Mortality rate plotted as boxplots against each class interval are shown in Figure 4.8. Visual inspection of the plots suggested a general decrease in mortality rate with increasing initial counts.

Analysed as a continuous variable by multivariable negative binomial regression, corrected for glasshouse and month, mortality rate was found to decrease significantly as the size of the population increased (IRR = 0.995, 95% CI = (0.992, 0.997); p<0.001, n = 97). The AIC and θ (95% CI) of the model were 699.2 and 3.88 (2.52, 5.24) respectively.

Analysed as a discrete variable, mortality rate was significantly decreased in the 301–350 class compared to the 51–100 class (p = 0.012). No significant differences were detected for any of the other classes of population size. A summary of the outputs of multivariable negative binomial regression for the association between mortality rate and initial counts (as a discrete factor), corrected for glasshouse and month, is presented in Table 4.4.
Figure 4.7. Scatterplots of initial counts against mortality rate (deaths/100 insects/week) for the overall population and for each glasshouse. The plot for the overall initial count data is shown in the top left, and the plots of sub-categorised data for each glasshouse are shown underneath.

Figure 4.8. Boxplot of initial counts (per glasshouse) against mortality rate. Initial counts have been subdivided into class intervals of 50 insects.
Table 4.4. Outputs of multivariable negative binomial regression to estimate the effect of population size (initial count) on mortality rate, corrected for glasshouse and month.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Levels</th>
<th>IRR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial count</td>
<td>51–100</td>
<td>1.00 (reference)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101–150</td>
<td>1.35 (0.81, 2.26)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>151–200</td>
<td>0.83 (0.49, 1.40)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>201–250</td>
<td>0.76 (0.42, 1.36)</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>251–300</td>
<td>0.79 (0.39, 1.60)</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>301–350</td>
<td>0.25 (0.08, 0.74)</td>
<td>0.013</td>
</tr>
</tbody>
</table>

IRR = incidence rate ratio; AIC = 700.19, θ (95% CI) = 4.20 (2.70, 5.69), n = 97.

4.3.5 The effect of local ambient temperature

Mortality rates plotted as scatterplots against monthly mean maximum daily temperature (°C) for the same month, and for one to five months prior are shown in Figure 4.9. A summary of outputs for each multivariable negative binomial analysis for the association between mortality rate and maximum temperate, correcting for sex and glasshouse, are shown in Table 4.5.
Figure 4.9. Scatterplots of mortality rate against monthly mean maximum daily temperature (°C) for time intervals from zero (same month) to five months prior to mortality. Lines of best fit with standard error (shaded area) were added using the LOESS (local regression) smoothing method.

Table 4.5. Outputs of multivariable negative binomial regression to estimate the effect of monthly mean daily maximum temperature (°C) on mortality rate, corrected for glasshouse and sex.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Factor</th>
<th>IRR (95% CI)</th>
<th>p-value</th>
<th>θ (95% CI)</th>
<th>AIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp (0 month prior)</td>
<td>1°C rise</td>
<td>1.08 (1.05, 1.10)</td>
<td>&lt;0.001</td>
<td>2.13 (1.59, 2.66)</td>
<td>1185.0</td>
</tr>
<tr>
<td>Temp (1 month prior)</td>
<td>1°C rise</td>
<td>1.09 (1.07, 1.12)</td>
<td>&lt;0.001</td>
<td>2.34 (1.72, 2.96)</td>
<td>1173.4</td>
</tr>
<tr>
<td>Temp (2 months prior)</td>
<td>1°C rise</td>
<td>1.04 (1.04, 1.09)</td>
<td>&lt;0.001</td>
<td>2.11 (1.57, 2.65)</td>
<td>1190.6</td>
</tr>
<tr>
<td>Temp (3 months prior)</td>
<td>1°C rise</td>
<td>1.03 (1.01, 1.06)</td>
<td>0.010</td>
<td>1.83 (1.39, 2.28)</td>
<td>1211.0</td>
</tr>
<tr>
<td>Temp (4 months prior)</td>
<td>1°C rise</td>
<td>0.99 (0.96, 1.01)</td>
<td>0.40</td>
<td>1.76 (1.34, 2.18)</td>
<td>1215.6</td>
</tr>
<tr>
<td>Temp (5 months prior)</td>
<td>1°C rise</td>
<td>0.95 (0.93, 0.98)</td>
<td>&lt;0.001</td>
<td>1.87 (1.42, 2.33)</td>
<td>1203.6</td>
</tr>
</tbody>
</table>

IRR = incidence rate ratio, n = 191.
Maximum temperatures from zero to two months prior to each examined month were associated with a highly significant but slight increase in mortality rate \((p < 0.001)\). Mortality rate against temperature one month prior had the strongest statistical association. Temperatures three months prior were associated with a slight increase in mortality rate with moderate significance \((p = 0.01)\). Temperatures four months prior were not associated with a statistically significant difference in mortality rate. Temperatures five months prior were significantly associated with a decrease in mortality rate.

4.3.6 Comparison of the post-mortem investigation period to other months

Mortality rates for the post-mortem period (period B) plotted against mortality rates for all other months (period A) as boxplots are shown in Figure 4.10. Multivariable negative binomial regression comparing the two time periods, corrected for sex, glasshouse and month, demonstrated the post-mortem investigation period was associated with a significantly decreased mortality rate \((\text{IRR} = 0.41, 95\% \text{ CI} = 0.35-0.94; p = 0.03, n = 191)\) compared to all other months. The AIC and \(\theta\) \((95\% \text{ CI})\) of the model were 1160.6 and \(\theta = 3.03\) \((2.16,3.89)\) respectively.

![Figure 4.10. Boxplot of mortality rate (deaths/100 insects/week) during the three-month post-mortem investigation period (period B) compared to all other months between 2010 and 2014 (period A).](image)
4.4 DISCUSSION

In this epidemiological investigation, mortality rates for the overall population were examined, and estimates derived for the effect of various factors on mortality rate. The minimum overall mortality rate (for either glasshouse) that occurred during the five-year period between 2010 and 2014 was 0.08 deaths/100 insect weeks and the maximum overall mortality rate was 8.92 deaths/100 insect weeks. The mean overall mortality rate was 2.51 deaths/100 insect weeks. Mortality rate distributions were found to be over-dispersed. Due to this skewed distribution, the median mortality rate was the preferred point estimate for this variable. The median overall mortality rate was calculated to be 2.04 deaths/100 insect weeks, which was comparable to the theoretically expected 1.92 deaths/100 insect weeks due to life expectancy alone.

Variables found to have statistically significant associations with mortality rate were glasshouse, month, year, temperature and time period. No statistically significant association was found between mortality rate and sex.

The median mortality rate for the month of July was calculated to be 1.08 deaths/100 insect weeks. Using July as a baseline and the monthly IRRs summarised in Table 4.2, the expected mortality rate for each month can be predicted. Mortality rates in future disease investigations can be compared against the expected mortality rate for each month.

Increased population size (increased stocking density) was found to be significantly associated with decreased mortality risk. As a discrete variable, mortality rate was only significantly decreased for the highest class for population size (300–350 insects per glasshouse), suggesting that a threshold population size of around 300 insects could be favourable for insect survival. Biological or husbandry-related factors did not provide an obvious explanation for why increased population size would result in a reduced mortality rate. Therefore, this association was considered to indicate that low mortality rates permit a high population size. Overstocking was dismissed as a significant risk factor for mortality, based on the range of population sizes included in this investigation.
Mortality rates were found to be slightly increased in GH6 compared to GH5. Further examination of the two glasshouses should be considered to look for possible factors that could explain this difference. Factors of interest include differences in: husbandry or environmental factors (e.g. temperature or humidity), distribution of pathogens, or conditions that could promote the growth of infectious organisms in the environment. Explanations for the difference in mortality rate could identify risk factors to address within each glasshouse and in the breeding facility as a whole.

Mortality rates were found to be strongly correlated with the months of the year, and demonstrated a seasonal pattern of fluctuation. This pattern occurred consistently for each year represented in the data. Peaks in mortality rate corresponded to the warmer summer months, and troughs corresponded to the colder winter months. February and March were associated with the greatest increases in risk of mortality. Compared to local temperature data, mortality rate was found to be strongly correlated with the monthly mean maximum daily temperatures up to 3 months prior to death, with high significance between 0 and 2 months prior to death. This finding suggests that the aetiology of a disease resulting in death could manifest as either an acute or chronic process following high external temperatures. If the disease process occurs over several months, the inciting cause for a peak death event, such as a heat wave, may not be obvious at the time when the deaths are finally observed.

These findings may indicate a direct effect of high temperatures on insect survival, an effect on bacterial growth or infection rate, an effect on disease susceptibility due to physiological stress associated with high temperatures, or other biological factors that fluctuate seasonally. Further investigation of internal glasshouse temperature data is required to assess the effect of internal temperature fluctuations on mortality rate, and to assess the degree to which internal glasshouse conditions are affected by external temperatures. Additionally, the effect of internal glasshouse humidity levels on mortality rate should also be investigated.

On one occasion during the current study (not represented in the current data), an unexpected cluster of mortalities was observed in the same two glasshouses after a two-day power outage in both glasshouses, which resulted in low temperatures and unregulated humidity levels (R. Cleave, pers. comm.). While not supported by statistical data, this
observation provided some further evidence that extremes or sudden changes in temperature or humidity may have an important role in the manifestation of disease. Large clusters of deaths were infrequently observed during the course of this study.

Mortality rate was found to vary significantly from year to year over the five-year period. Additional years of data would need to be examined to determine if this pattern is consistent over a longer time frame, and to establish what factors, such as annual climate patterns or variations in husbandry practices, might contribute to this variation.

Finally, when the post-mortem investigation period at the beginning of this current study (August to October 2014 inclusive) was compared with the rest of the months in the data, the mortality rate was found to be decreased. This may have been partly explained by the effects of month and temperature, since the investigation was conducted during months of the year associated with lower mortality rates and lower temperatures. In light of this, the findings of the post-mortem investigation may need to be interpreted with the knowledge that they represent a ‘low mortality’ period of time.

Due to the limited information in the original data, some major assumptions were made in the initial calculations for mortality rate. All mortalities and moves were assumed to have occurred either exactly in the middle of the month, or exactly ¾ of the days into the month. However, observations suggest that mortalities are more likely to be spread over the course of the month. Mortality rate estimates may be accurate for the average over the month, but daily and weekly mortality rates could be more variable than what has been estimated. For months when repopulation of a glasshouse occurred, all insects were assumed to have been added during a single event, exactly in the middle of the month. However, insects could have been added or removed in multiple, scattered events over the course of the month. Therefore, the true mortality rates during those months were likely to differ from the calculated mortality rates. The numbers of insects moved in and out each month were derived from the numbers not accounted for by mortality counts, assuming both population size and mortality counts were accurate. Any errors in counting would have resulted in at least two errors in the mortality rate equation for that month.
Juvenile insects were not included in this investigation due to lack of available data. The collection of mortality data for juvenile insects is currently hindered by their excellent camouflage, small body size and the possibility of predation by invertebrate pests prior to discovery. Development of additional methods to accurately count mortalities in the juvenile group is required in order to gain any information about mortality rate and risk factors for mortality in that age class.

4.5 CONCLUSION

In this epidemiological investigation, the mortality rates and risk factors were characterised for two glasshouses that house adult and sub-adult *D. australis* in the captive breeding colony. Baseline median mortality rates were estimated for the population and incidence risk ratios were estimated for each identified risk factor, providing a reference for future disease outbreak investigations in these two glasshouses. Due to the limitations in this investigation, extrapolation of the current findings to other glasshouses, age classes, or populations of *D. australis* should be performed with care.
CHAPTER 5. PFGE TYPING OF SERRATIA MARCESCENS ISOLATED FROM STICK INSECTS AT POST-MORTEM

5.1 INTRODUCTION

In the current study, PFGE typing was performed to assess the degree of molecular relatedness of *S. marcescens* isolates cultured from *D. australis* during post-mortem examination, those collected from samples of the insects’ environment, and those isolated from samples collected following experimental infection. The purpose of this investigation was to identify common strains of *S. marcescens* from the stick insect colony, to infer the likelihood of pathogenicity in the isolates, and to use molecular typing techniques to compare isolates that were cultured from the different sources.

This chapter describes the specific methodology and results of two PFGE experiments conducted to compare and sub-type isolates cultured from the haemolymph of insects at post-mortem examination, followed by the selection of the candidate strain for the experimental infection trial. The PFGE typing of isolates cultured during the environmental screening experiment and the experimental infection trial are detailed in Chapter 7.

5.2 MATERIALS AND METHODS

Two PFGE analyses were performed for *S. marcescens* isolates cultured from post-mortem haemolymph samples. The first analysis was conducted mid-way through the post-mortem sampling period, and included isolates from both pure and mixed cultures. The second PFGE analysis was conducted at the completion of the sampling period, and included only pure culture isolates. Twenty-four isolates were included in each analysis, with some isolates represented in both analyses.

The criteria for selection of isolates is described in Section 5.2.1. The PFGE methods used for each experiment were as described in Chapter 2, except where otherwise specified.
5.2.1 Selection of isolates and modifications to methodology

A total of 24 isolates were represented in each analysis, limited by the number of wells in the PFGE gel. In the initial PGFE analysis, 22 isolates were selected from a total of 28 S. marcescens isolates cultured from haemolymph samples. Two control isolates of S. marcescens from the preliminary investigation, VW347 and VW348, representing the dominant PFGE profile and the outlier profile respectively, were also included (J. Allen, 2014, unpublished data).

The selection of the 22 isolates was based on the following criteria:

1) all 17 available pure culture isolates were included as a priority;
2) for isolates subcultured from two-genera mixed cultures, the 5 with the shortest death-to-sampling period were included;
3) no isolates from mixed cultures containing more than two genera were included.

A summary of the isolates selected for the initial PFGE analysis is presented in Table 5.1, including the date of the post-mortem examination and date of sample collection (PME date), the age class, sex and glasshouse location of each insect sampled, the death-sampling interval, and whether the isolate had been cultured in pure or mixed culture.

At the completion of the post-mortem investigation, a total of 24 pure culture isolates of S. marcescens had been isolated from haemolymph samples. A second PFGE experiment was conducted, including 22 of those S. marcescens isolates, all from pure cultures. The two control isolates, VW347 and VW348, were again included. The two isolates with the greatest time death-sampling interval were excluded from the analysis.

A summary of the isolates selected for the second PFGE analysis is presented in Table 5.2, including the date of the post-mortem examination and date of sample collection (PME date), the age class, sex and glasshouse location of each insect sampled, the death-sampling interval (days), and whether the isolate had been cultured in pure or mixed culture.

Lanes of the resultant gel image that featured heavily smeared bands were cropped out using Adobe® Photoshop CC graphics software (2015 version). Restriction endonuclease digestion and electrophoresis was repeated for isolates, AM923 and AM1004. For each isolate, the lane
featuring the least band smearing was included in the final gel image and used for dendrogram analysis.

Table 5.1. Summary of *Serratia marcescens* isolates included in the initial PFGE analysis.

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>PME date</th>
<th>Insect age class</th>
<th>Insect sex</th>
<th>GH</th>
<th>Death-sampling interval (days)</th>
<th>Pure culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>VW347</td>
<td>06/02/14</td>
<td>Sub-adult</td>
<td>–</td>
<td>5</td>
<td>–</td>
<td>Y</td>
</tr>
<tr>
<td>VW348</td>
<td>06/02/14</td>
<td>Sub-adult</td>
<td>–</td>
<td>5</td>
<td>–</td>
<td>Y</td>
</tr>
<tr>
<td>SM810</td>
<td>10/08/14</td>
<td>Sub-adult</td>
<td>M</td>
<td>5</td>
<td>3</td>
<td>N</td>
</tr>
<tr>
<td>JU803a</td>
<td>03/08/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>10</td>
<td>Y</td>
</tr>
<tr>
<td>JU810n1</td>
<td>10/08/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td>JU810n2</td>
<td>20/08/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td>AF820</td>
<td>20/08/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>JU820</td>
<td>20/08/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>JF824a</td>
<td>24/08/14</td>
<td>Juvenile</td>
<td>F</td>
<td>2</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AF828</td>
<td>28/08/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AM830</td>
<td>30/08/14</td>
<td>Adult</td>
<td>M</td>
<td>1</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>JU830</td>
<td>30/08/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>AM831</td>
<td>31/08/14</td>
<td>Adult</td>
<td>M</td>
<td>5</td>
<td>1</td>
<td>N</td>
</tr>
<tr>
<td>AF906</td>
<td>06/09/14</td>
<td>Adult</td>
<td>F</td>
<td>6</td>
<td>2</td>
<td>N</td>
</tr>
<tr>
<td>SM911</td>
<td>10/09/14</td>
<td>Sub-adult</td>
<td>M</td>
<td>2</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AM911</td>
<td>11/09/14</td>
<td>Adult</td>
<td>M</td>
<td>5</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>AM923</td>
<td>23/09/14</td>
<td>Adult</td>
<td>M</td>
<td>1</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td>JM925</td>
<td>5/09/14</td>
<td>Juvenile</td>
<td>M</td>
<td>2</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AF925</td>
<td>25/09/14</td>
<td>Adult</td>
<td>F</td>
<td>6</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>JU929</td>
<td>29/09/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AM1003</td>
<td>03/10/14</td>
<td>Adult</td>
<td>M</td>
<td>1</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AM1004</td>
<td>04/10/14</td>
<td>Adult</td>
<td>M</td>
<td>6</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>AF1006</td>
<td>06/10/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AF1007</td>
<td>07/10/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>0</td>
<td>Y</td>
</tr>
</tbody>
</table>
Table 5.2. Summary of *Serratia marcescens* isolates included in the second PFGE analysis.

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>PME date</th>
<th>Insect age class</th>
<th>Insect sex</th>
<th>GH</th>
<th>Death-sampling interval (days)</th>
<th>Pure culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>VW347</td>
<td>06/02/14</td>
<td>Sub-adult</td>
<td>–</td>
<td>5</td>
<td>–</td>
<td>Y</td>
</tr>
<tr>
<td>VW348</td>
<td>06/02/14</td>
<td>Sub-adult</td>
<td>–</td>
<td>5</td>
<td>–</td>
<td>Y</td>
</tr>
<tr>
<td>JU810n1</td>
<td>10/08/14</td>
<td>Sub-adult</td>
<td>M</td>
<td>2</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td>AF820</td>
<td>20/08/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>JU820</td>
<td>20/08/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>JF824a</td>
<td>24/08/14</td>
<td>Juvenile</td>
<td>F</td>
<td>2</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AF828</td>
<td>28/08/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>JU830</td>
<td>30/08/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>SM911</td>
<td>11/09/14</td>
<td>Sub-adult</td>
<td>M</td>
<td>2</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AM923</td>
<td>23/09/14</td>
<td>Adult</td>
<td>M</td>
<td>1</td>
<td>3</td>
<td>Y</td>
</tr>
<tr>
<td>JM925</td>
<td>25/09/14</td>
<td>Juvenile</td>
<td>M</td>
<td>2</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AF925</td>
<td>25/09/14</td>
<td>Adult</td>
<td>F</td>
<td>6</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>JU929</td>
<td>29/09/14</td>
<td>Juvenile</td>
<td>–</td>
<td>2</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AM1003</td>
<td>03/10/14</td>
<td>Adult</td>
<td>M</td>
<td>1</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AM1004</td>
<td>04/10/14</td>
<td>Adult</td>
<td>M</td>
<td>6</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>AF1006</td>
<td>06/10/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AF1007</td>
<td>07/10/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AF1008</td>
<td>08/10/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AF1013</td>
<td>13/10/14</td>
<td>Adult</td>
<td>F</td>
<td>5</td>
<td>2</td>
<td>Y</td>
</tr>
<tr>
<td>AF1016</td>
<td>16/10/14</td>
<td>Adult</td>
<td>F</td>
<td>6</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AF1020</td>
<td>20/10/14</td>
<td>Adult</td>
<td>F</td>
<td>6</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>AF1021</td>
<td>21/10/14</td>
<td>Adult</td>
<td>F</td>
<td>7</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>AM1023</td>
<td>23/10/14</td>
<td>Adult</td>
<td>M</td>
<td>6</td>
<td>1</td>
<td>Y</td>
</tr>
<tr>
<td>SM1025</td>
<td>25/10/14</td>
<td>Sub-adult</td>
<td>F</td>
<td>6</td>
<td>2</td>
<td>Y</td>
</tr>
</tbody>
</table>
5.2.2 Dendrogram construction and type analysis

Dendrograms were constructed from the gel images using GelJ software (version 2.0) (Heras et al., 2015), as described in Chapter 2. Strain types were assigned based on interpretation of the constructed dendrograms.

For the initial experiment, a marker lane was imported from a secondary gel image, to account for a missing marker lane in the original image. The secondary gel image included lanes showing a Lambda Ladder PFG Marker (New England BioLabs® Inc., Ipswich, Massachusetts, USA) directly next to isolate AM923, which was represented in both images. Using AM923 as a reference, the secondary image was cropped and imported next to the main gel image using Adobe® Photoshop CC (2015 version). The two parts of the image were then aligned such that the bands for each lane for AM923 matched exactly in position. Band analysis and dendrogram construction were performed using the combined image, with the imported marker used as a reference for fragment size.

The final designated sub-types were based on the results of the second analysis, which included only isolates of S. marcescens isolated pure culture.

5.2.3 Statistical analysis

To assess for any relationship between insect factors (age class, sex and glasshouse location) and bacteriological factors (death-sampling interval and culture purity) and designated stain type, a Pearson’s Chi-squared test for independence was performed between each factor and each major type, and between each factor and each subtype. Statistical analysis was performed using the statistical software, R (version 2.14.2), with the statistical package MASS (version 7.3-17). For the second PFGE analysis, no statistical test was performed for the effect of culture purity, since all included isolates were from pure cultures. A total of ten Chi-squared tests were performed for the first PFGE analysis, and eight Chi-squared tests were performed for the second PFGE analysis. P-values <0.05 were considered statistically significant.
5.3 RESULTS

The results of the first PFGE analysis, including a total of 24 pure and mixed culture post-mortem haemolymph isolates of *S. marcescens*, with the 2 control isolates, are shown in Figure 5.1. The dendrogram constructed from the results of the first PFGE analysis is shown in Figure 5.2.

The results of the second PFGE analysis, including 24 pure culture isolates of *S. marcescens*, with the 2 control isolates, are shown in Figure 5.3. The dendrogram generated from the results of the second PFGE analysis is shown in Figure 5.4.

![Figure 5.1](image.png)

**Figure 5.1.** Results of the initial PFGE analysis for genomic DNA extracted from *Serratia marcescens* isolated from haemolymph at post-mortem examination (n=22). Control isolates VW347 and VW348 from the preliminary disease investigation were included.
Figure 5.2. Dendrogram based on the initial PFGE analysis that featured *Serratia marcescens* isolated from haemolymph at post-mortem examination (n=22). Control isolates VW347 and VW348 from the preliminary disease investigation were included. The dendrogram was constructed using the Dice similarity method with UPGMA linkage and 3% tolerance. Coloured boxes indicate those isolates designated as type A1 (blue), type A2 (green), type B1 (orange) and type B2 (red). Isolate AM1003 was designated as type A.
Figure 5.3. Results of the second PFGE gel for genomic DNA extracted from *Serratia marcescens* isolated from haemolymph at post-mortem examination (n=22). Control isolates VW347 and VW348 from the preliminary disease investigation were included.
Figure 5.4. Dendrogram based on the second PFGE analysis that featured *S. marcescens* isolated from haemolymph at post-mortem (n=22). Control isolates VW347 and VW348 from the preliminary disease investigation were included. The dendrogram was constructed using the Dice similarity method with UPGMA linkage and 3% tolerance. Coloured boxes indicate those isolates designated as type A1 (blue), type A2 (green) and type B (orange). Isolate AM1003 was designated as type A.
Based on the dendrograms for each PGFE analysis (Figure 5.2 and Figure 5.4), two major types were identified for *S. marcescens* isolates cultured from haemolymph at post-mortem examination. The major types were designated ‘type A’ and ‘type B’, separated at approximately 74% similarity. Type A was designated as the dominant type, represented by 20 out of 24 isolates (83%) in the first analysis, and by 21 out of 24 isolates (88%) in the second analysis. The remaining isolates in each analysis were designated as type B.

In each analysis, two subtypes of type A (type A1 and type A2) were identified, separated at approximately 95% similarity, along with a single outlier in isolate AM1003. In the first analysis (Figure 5.1 and Figure 5.2), 13 out of 20 type A isolates (65%) represented the dominant A1 subtype, while 6 isolates (30%) represented the lesser A2 subtype. In the second analysis (Figure 5.3 and Figure 5.4), 12 out of 21 type A isolates (57%) represented the A1 subtype, while 8 isolates (38%) represented the lesser A2 subtype. Two equally dominant subtypes of type B, type B1 (isolates VW348 and JU03a) and type B2 (AF903 and AM1004) were evident in the first analysis, separated at approximately 97% similarity (Figure 5.2), but only one group of type B isolates were identified in the second analysis (Figure 5.4).

Based on the second analysis (Figure 5.3 and Figure 5.4), no band differences were detected between isolates within each subtype of type A. Type A1 isolates could be distinguished from type A2 isolates by a four-band difference. The three isolates that represented type B were genetically indistinguishable from one another. Type B isolates showed at least 10 band differences compared to type A isolates. In both analyses, the control isolates VW347 and VW348 were typed as type A1 and type B respectively (Figure 5.2 and Figure 5.4).

Using Pearson’s Chi-squared test, the number of days between insect death and haemolymph sampling was found to have a statistically significant effect on strain type in the first analysis at both the major type level (p = 0.030) and at the subtype level (p = 0.005), but no significant effect was detected in the second analysis. Glasshouse location had a statistically significant effect on strain lineage at only the subtype level (p = 0.027) in the first analysis, but not in the second analysis. No statistically significant effect of insect age class, insect sex, or sample purity was detected.
5.4 DISCUSSION

Isolates that featured in both analyses typed consistently for major type between the two analyses. However, additional subtypes of type B were observed in the first analysis, which included isolates from mixed cultures or associated with relatively long death-to-sampling intervals, compared to the second analysis. The consistency of typing results between the two analyses suggested that the current PFGE method was reliable and replicable for *S. marcescens* isolates in this study.

The death-sampling interval and glasshouse location of the insect at the time of death were the only factors identified to have statistically significant effects on typing results for *S. marcescens* isolates in the first analysis. However, those effects were not significant in the second analysis, which included only pure culture isolates. Since the sample size of each data set was small (n=24), and data for insect sex and/or death-sampling interval was missing for some isolates, further evaluation of typing results for a larger data set would be required before extrapolating these findings to all *S. marcescens* isolated from insects in the facility.

In the initial analysis (Figure 5.2), both pure and mixed culture isolates of *S. marcescens* were included within each major type, and no distinction in typing pattern was observed between pure and mixed culture isolates. Compared to the second PFGE analysis (Figure 5.4), in which isolates from mixed cultures or delayed post-mortem sampling were excluded, the initial analysis demonstrated a more complex clustering hierarchy for isolates within each major type. Two additional levels of clustering were observed within Type A and one additional level was observed within Type B, compared to the second analysis.

Within each subtype of type A, no band differences were detected, indicating that the isolates were genetically indistinguishable from one another (Tenover et al., 1995). The four-band difference between type A1 isolates and type A2 isolates could be explained by two independent genetic events, such as two DNA deletion events or two DNA insertion events, and suggested possible genetic-relatedness, but limited epidemiological-relatedness between the two subtypes. Such a relationship between two subtypes could be consistent with an extended outbreak situation (Tenover et al., 1995). Thus, in this investigation, type
A1 and type A2 were considered to be two genetically-related strains of *S. marcescens*. The type A1 strain was represented most frequently and was implicated as the most likely outbreak strain.

Type B isolates were considered to represent a single strain of *S. marcescens*, based on the findings of the second analysis (Figure 5.4) which demonstrated no detectable band differences between the three isolates representing type B. A difference of at least 10 bands between type A and type B isolates suggested that the two types were genetically and epidemiologically unrelated to one another (Tenover et al., 1995).

The typing of control isolates VW347 and VW348 from the preliminary investigation, as type A1 and type A2 respectively was consistent with the findings of the PFGE analysis conducted during the preliminary disease investigation, in which VW347 was representative of the dominant type profile (4 out of 5 isolates) and VW348 represented a single outlier (J. Allen, unpublished data).

Other molecular typing methods to study *S. marcescens* outbreaks include random amplified polymorphic DNA PCR (RAPD-PCR) (Hejazi et al., 1997; Enciso-moreno et al., 2004; Muellner et al., 2011), multilocus sequence typing (MLST) (Batah et al., 2015), repetitive-sequence-based PCR (Ligozzi et al., 2010), arbitrarily-primed PCR (Yoon et al., 2004) and plasmid analysis (Gargallo-violat, 1989). These typing methods were beyond the scope of the current investigation, but could be used to further define *S. marcescens* types in future investigations.

While PFGE studies have previously been performed for isolates of *S. marcescens* involved in human disease outbreaks, at the time of writing, no reports of comparable PFGE studies of *S. marcescens* were available for any taxonomically-similar insect species. Further research, including additional PFGE and other molecular typing studies, is required to determine the relatedness of the *S. marcescens* strains in the current study to other insect strains, human strains, and plant strains of *S. marcescens*. 
5.5 CONCLUSION

Based on the findings of this PGFE investigation, the type A1 strain of *S. marcescens*, isolated from the haemolymph of dead and moribund *D. australis*, was implicated as the most likely outbreak strain. The related type A2 strain of *S. marcescens* was also identified. Isolate AM923, representative of the type A1 strain and isolated from haemolymph in pure culture, was selected as the inoculating strain for the experimental infection trial (Chapter 7). PFGE was demonstrated to be a suitable molecular typing tool for discriminating between *S. marcescens* isolates in the current disease investigation.
CHAPTER 6. ENVIRONMENTAL SCREENING

6.1 INTRODUCTION

*S. marcescens* has been previously described as an opportunistic pathogen in other species, and has been detected from environmental sources including water, soil and plant sources (Hejazi and Falkiner, 1997; Grimont and Grimont, 2006). However, attempts to culture *S. marcescens* from frass (insect faeces) and nest boxes in the *D. australis* captive breeding facility at Melbourne Zoo prior to the current study had been unsuccessful (S. Frith, 2014, pers. comm.) Whether *S. marcescens* existed within the facility environment, and the degree of relatedness between potential environmental isolates and insect isolates remained unknown.

To investigate possible environmental sources of *S. marcescens* within the breeding facility, and to develop potential environmental screening techniques for future investigations, a pilot study was performed. Samples were collected from two glasshouses within the facility and screened for the presence of *S. marcescens* using bacteriological culture methods.

6.2 METHODS

6.2.1 Sample collection

Samples were collected from two glasshouses of the breeding facility, glasshouse 5 (GH5) and glasshouse 6 (GH6), which each housed free-ranging adult and sub-adult stick insects. All samples were collected within a 4-hour period, during February 2015. From each glasshouse, four types of samples were collected: frass, drinking water, swabs of nest box surfaces, and swabs of floor surfaces. Clean, disposable gloves were worn throughout sampling procedures, and new gloves were worn during the collection of each sample.

The number of each sample type was dependent on each glasshouse set-up at the time of sampling. Nest box surfaces and frass were sampled at every nest box station, and all drinking water dishes were sampled. Nest box stations each consisted of either one or two nest boxes.
on a raised platform, and a potted feed plant. GH5 contained six nest box stations: five stations with single nest boxes and one station with two nest boxes in direct contact. GH6 contained only four stations, each with two nest boxes in direct contact. Two filled drinking water dishes were present on the floor of each glasshouse. One floor swab sample was collected from each glasshouse. The numbers of samples for each sample type and from each glasshouse are summarised in Table 6.1.

**Table 6.1.** Summary of environmental sample types and sample quantities collected from glasshouse 5 and glasshouse 6.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Quantity</th>
<th>Glasshouse 5</th>
<th>Glasshouse 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frass</td>
<td>20-30 pieces</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Drinking water</td>
<td>10 mL</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Nest box surface</td>
<td>1 swab</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Floor surface</td>
<td>1 swab</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>

A swab sample of each nest box surface was collected by wiping the outer surfaces of the lid and four sides using a 7.5 cm x 7.5 cm piece of sterile square gauze swab, pre-moistened with sterile 0.9% NaCl solution (‘saline’). For stations with two nest boxes in direct contact, the two boxes were treated as a single unit and sampled together. From each water dish, 10 mL of water was collected using a micropipette. A pooled frass sample, consisting of 20 to 30 pieces of frass, was collected from each station, including frass from inside the nest box and from the floor directly below. For floor samples, a 1 m x 1 m square of floor surface, visibly free of frass and plant material, was swabbed using a 7.5 cm x 7.5 cm piece of gauze, pre-moistened with sterile saline, as described for nest box sampling. Each sample was immediately placed into a sterile plastic tube.

All samples were transported to the laboratory immediately following completion of sampling. Each sample was initially processed on the same day as collection, with the remaining portion of each sample stored at 4°C until further processing.
Each sample was assigned a name corresponding to the glasshouse, sample type, and numerical order of the samples. For example, sample ‘5 water 1’ was the first water sample collected from GH5, and ‘6 box 2’ was the second nest box sample collected from GH6. Isolates of *S. marcescens* cultured from the samples were named according to the sample name, and the colour of the colonies.

### 6.2.2 Sample processing and plating

For each frass sample, frass pellets were manually homogenised into a fine sediment using a sterilised steel spatula. A sample (0.1 g) of the homogenised frass was suspended into 10 mL of sterile saline, resulting in a 10 mg/mL frass suspension. Each suspended frass sample was left upright in a rack for 30 minutes to allow sedimentation of frass particles. Nest box and floor swab samples were suspended by adding 10 mL of sterile saline and agitating.

For each suspended test sample, an aliquot (100 µL) of supernatant was spread directly onto LB/CHX agar, prepared as described in Chapter 2. Each water sample was vortexed before an aliquot (100 µL) was spread directly onto LB/CHX agar. All cultures were incubated aerobically for 18 hours at 37°C.

In a second round of sample processing, conducted 6 days after sample collection, frass, nest box and floor samples were cultured using two higher dilution factors. For each frass sample, another 0.1 g of homogenised frass was suspended in 10 mL of sterile saline, then left for 30 minutes as previously described. For floor and nest box swab samples, each previously suspended sample was agitated again prior to dilution. An aliquot (100 µL) of supernatant was collected from each sample and serial diluted to $10^{-1}$ and $10^{-2}$ dilutions in sterile saline. An aliquot (100 µL) of each final dilution was then spread onto LB/CHX agar. For frass, the two dilutions corresponded to frass concentrations of 1 mg/mL and 0.1 mg/mL respectively. Water samples were each agitated and spread directly onto LB/CHX agar without dilution. All plates were incubated for 18 hours at 37°C.
6.2.3 Subculture and colony testing

Colonies were manually selected from LB/CHX agar and spot-inoculated onto DNase agar (Media Preparation Unit, the University of Melbourne, Parkville, VIC) to test for DNase activity. From each culture, at least ten non-pigmented, mucoid, opaque to semi-translucent colonies, and all red, mucoid colonies were selected for DNase testing. Colonies were inoculated onto DNase agar at evenly-spaced intervals. Previously identified *S. marcescens* isolates, AM923 and AM1004, from the post-mortem investigation were also spot-inoculated onto separate DNase agar plates for comparison. DNase agar plates were incubated aerobically for 18-24 hours at 37°C, before the colonies were examined for growth and DNase activity. For frass, floor and nest box samples, colonies were selected only from the plates corresponding to $10^{-2}$ sample dilution. For water samples, colonies were selected only from the cultures from the second round of processing. For nest box samples, only colonies from one sample, ‘6 box 1’, were subjected to DNase testing due to loss of the remaining samples.

To confirm DNase positive colonies as *S. marcescens*, the bacteria were examined microscopically following Gram staining, and tested biochemically as described in Chapter 3. For each sample, a single colony for each non-pigmented and each pigmented (red) isolate of *S. marcescens* was subcultured onto SBA, and finally stored at -80°C for further experiments, using the methods described in Chapter 2.

6.2.4 PFGE analysis

Five environmental isolates of *S. marcescens* were included in a PFGE analysis. The results were compared against the twenty-four pure-culture, post-mortem haemolymph isolates that were previously analysed using PFGE analysis (Chapter 5). PFGE analysis and dendrogram construction were performed according to the methods described in Chapter 2, and strain subtypes were designated according to the dendrograms constructed in the second analysis of the post-mortem PFGE investigation (Chapter 5).
6.3 RESULTS

6.3.1 Culture results
For each of the four water samples, light to moderate mixed bacterial growth was observed on LB/CHX agar. For nest box, floor and frass samples, moderate to heavy growth was observed at the $10^{-2}$ dilution. Most colonies appeared mucoid and non-pigmented, ranging from opaque to translucent, while a smaller number of mucoid, brightly pigmented (red), opaque colonies were observed for some samples.

The numbers of non-pigmented and pigmented colonies that were selected for DNase testing, the number of colonies of each colour that grew on DNase agar, and the number of DNase positive colonies for each sample are summarised in Table 6.2. Only 4 out of 135 (3%) of non-pigmented isolates that grew on DNase agar demonstrated DNase activity, while all 18 of the pigmented isolates that grew on DNase agar demonstrated DNase activity.
Table 6.2. Results of DNase testing of selected colonies cultured from frass, water, floor and nest box samples.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>GH</th>
<th>Sample name</th>
<th>Tested</th>
<th>Grown</th>
<th>DNase positive</th>
<th>Tested</th>
<th>Grown</th>
<th>DNase positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frass</td>
<td>5</td>
<td>5 frass 1</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5 frass 2</td>
<td>10</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5 frass 3</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5 frass 4</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5 frass 5</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5 frass 6</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6 frass 1</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6 frass 2</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6 frass 3</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6 frass 4</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Water</td>
<td>5</td>
<td>5 water 1</td>
<td>10</td>
<td>6</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5 water 2</td>
<td>10</td>
<td>7</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6 water 1</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6 water 2</td>
<td>13</td>
<td>8</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Floor</td>
<td>5</td>
<td>5 floor 1</td>
<td>20</td>
<td>15</td>
<td>0</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6 floor 1</td>
<td>23</td>
<td>12</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Nest box</td>
<td>6</td>
<td>6 box 1</td>
<td>13</td>
<td>8</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>199</td>
<td>135</td>
<td>4</td>
<td>20</td>
<td>18</td>
<td>18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A total of seven isolates of *S. marcescens* from four different environmental sources were identified, including two non-pigmented isolates and five pigmented isolates. For each isolate, the assigned isolate name, sample name, pigmentation phenotype, glasshouse and sample type are summarised in Table 6.3. Of those, six isolates were subcultured and stored, while the remaining pigmented isolate, 5Flr, was lost prior to subculture.
Table 6.3. Seven isolates of *Serratia marcescens* identified from culture of water, nest box, frass and floor samples from glasshouse 5 and glasshouse 6.

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Sample name</th>
<th>Pigmentation</th>
<th>Glasshouse</th>
<th>Sample type</th>
</tr>
</thead>
<tbody>
<tr>
<td>5W2w</td>
<td>5 water 2</td>
<td>Non-pigmented</td>
<td>5</td>
<td>Water</td>
</tr>
<tr>
<td>6F1w</td>
<td>6 floor 1</td>
<td>Non-pigmented</td>
<td>6</td>
<td>Floor</td>
</tr>
<tr>
<td>5F1r</td>
<td>5 floor 1</td>
<td>Pigmented</td>
<td>5</td>
<td>Floor</td>
</tr>
<tr>
<td>6W2r</td>
<td>6 water 2</td>
<td>Pigmented</td>
<td>6</td>
<td>Water</td>
</tr>
<tr>
<td>6B1r</td>
<td>6 box 1</td>
<td>Pigmented</td>
<td>6</td>
<td>Nest box</td>
</tr>
<tr>
<td>6F1r</td>
<td>6 floor 1</td>
<td>Pigmented</td>
<td>6</td>
<td>Floor</td>
</tr>
<tr>
<td>6Fr4r</td>
<td>6 frass 4</td>
<td>Pigmented</td>
<td>6</td>
<td>Frass</td>
</tr>
</tbody>
</table>

6.3.2 PFGE results

The dendrogram comparing PFGE results for five environmental isolates of *S. marcescens*, 5W2w, 6F1w, 6W2r, 6B1r and 6Fr4r, compared to the previous post-mortem haemolymph isolates, is shown in Figure 6.1. Based on the strain types designated previously in Chapter 5, the two non-pigmented environmental isolates, 5W2w and 6F1w, were shown to group as type A2. The three pigmented environmental isolates, 6W2r, 6B1r and 6Fr4r, were shown to group as type B2. Type A and type B strains were separated at 73% similarity, type A1 and type A2 strains were separated at 96% similarity, while type B1 and B2 strains were separated at 76% similarity.
Figure 6.1. Dendrogram comparing the PFGE analysis results between *Serratia marcescens* isolated from the environmental screening investigation (n = 5) to pure-culture haemolymph isolates from the post-mortem investigation (n = 22). Control isolates VW347 and VW348 from the preliminary disease investigation were included. The dendrogram was constructed using the Dice similarity method with UPGMA linkage and 3% tolerance. Coloured boxes indicate those isolates designated as type A1 (blue), type A2 (green), type B1 (orange) and type B2 (red). Isolate AM1003 was designated as type A.
6.4 DISCUSSION

Seven isolates of *S. marcescens* were identified from culture of environmental samples, including both non-pigmented and pigmented isolates. Five of those isolates were cultured from samples collected in GH6, while the other two were collected from GH5. *S. marcescens* was detected in each of the four samples types examined: drinking water, frass, nest box surfaces and floor surfaces. Pigmented isolates were isolated from all four sample types, while non-pigmented isolates were isolated from water and floor samples only. Both pigmented and non-pigmented isolates were detected in each glasshouse. Both non-pigmented and pigmented *S. marcescens* isolates were detected from the floor of GH6. For the other five samples that contained *S. marcescens*, only a single pigmentation phenotype was isolated. Pigmented colonies were also observed in the first round of cultures for 5 out of 10 nest boxes, including samples from both glasshouses, however the cultures were lost prior to testing of the colonies to confirm species identity. The current findings suggest that pigmented *S. marcescens* may be widespread in the glasshouse environment, while the distribution of non-pigmented in the environment appears to be more limited. No evidence was found to suggest that non-pigmented *S. marcescens* was present in the frass of insects at the time of this investigation.

Since colonies were manually selected for testing, non-pigmented colonies of *S. marcescens* may have been overlooked among the numerous, morphologically-similar non-pigmented colonies. Only a small proportion (3%) of the non-pigmented colonies that grew on DNase agar, and only 2% of the total number of non-pigmented colonies tested, demonstrated DNase activity, consistent with *Serratia* sp. (Table 6.2). This finding indicated that the majority of non-pigmented colonies represented bacteria other than *Serratia* sp. However, further testing to identify those bacteria was not performed. Colonies that did not grow on DNase agar may have represented bacteria that were unable to utilise the media, or a failure to inoculate the media with live bacteria. Pigmented colonies were much easier to identify, compared to non-pigmented colonies, due to their striking red colour, and were less likely to have been missed during colony selection. Selection bias in favour of pigmented *S. marcescens* colonies is likely to have resulted in an over-representation of pigmented isolates compared to non-pigmented isolates. The sensitivity of detection for non-pigmented *S.
marcescens colonies was also likely to have varied between plates of different colony densities due to the limited number of colonies selected for testing. Although a consistent pigmentation phenotype was observed at 37°C for each strain during this investigation, the culture of isolates under a range of different temperatures and growth conditions is required to conclusively define the pigmentation characteristics of each strain. Molecular comparison of pigmented and non-pigmented isolates is required to determine if the differences in pigmentation phenotype correspond to genetic differences.

PFGE analysis showed that none of the five environmental S. marcescens isolates were representative of the suspected outbreak strain, S. marcescens type A1, based on the previous PFGE investigation of post-mortem isolates (Figure 6.1). The two non-pigmented isolates were identified as type A2, which had previously been isolated from dead stick insects. S. marcescens type A2 may represent an environmental strain that is potentially associated with insect mortality, and a potential epidemiological origin of the type A1 strain. Alternatively, the type A2 isolates may have represented bacteria that had been recently shed by infected insects.

All pigmented isolates were identified as S. marcescens type B but were not closely related to the type B isolates previously isolated from insects at post-mortem examination. Consequently, the pigmented environmental isolates were assigned as type B2 and were not considered to be associated with insect disease.

Since only one colony of each pigmentation phenotype was selected for subculture and PFGE from each S. marcescens positive sample, the relatedness of those colonies to the remaining S. marcescens colonies was not determined. PFGE analysis of a greater number of colonies from each sample should be considered in future studies, to investigate the possibility of multiple strains of S. marcescens coexisting within a single source.

The detection of S. marcescens type A2 in only 1 out of 4 drinking water samples suggests that the common water source (mains water) was unlikely to be a major source of disease-associated S. marcescens virulent was unlikely, although further testing samples from water dishes and directly from the mains source is required to provide further evidence for this
statement. Alternative explanations for the presence of *S. marcescens* in the water sample include: insects contaminating the water dish while drinking or walking through the dish, frass or plant material falling into the water from the nest box stations above, previous contamination of the dish itself, or contamination of the collected sample.

For water samples, undiluted samples were appropriate for plating directly. For the suspension of frass, nest box and floor samples, a $10^{-2}$ dilution factor was found to produce the most suitable colony density for sampling isolated colonies. Further dilution to $10^{-3}$ or plating of a smaller aliquot could be considered in future experiments to further aid colony selection. However, further dilution could also prevent detection of *S. marcescens* at low concentrations. For nest box and floor samples collected using a gauze swab, the optimal dilution factor is expected to vary between samples, depending on the quantity of organic matter collected on each swab. The manual homogenisation method for frass was simple and easy to perform with minimal equipment. However, the final particle size was difficult to control using this technique, which could have affected the bacterial concentrations of the suspended samples.

Due to the prolonged storage of samples at 4°C during delayed processing, the potential for overgrowth of certain bacterial populations could not be excluded in this investigation. Immediate storage of samples at -20°C would be preferable in future experiments, unless sample processing could be performed immediately.

During this pilot study, the manual selection of individual colonies for testing was found to be extremely time-consuming for a single investigator. Only a small number of colonies could be tested for each sample, and only a limited number of samples could be processed each day, which resulted in delayed sample processing. To enable more efficient detection of *S. marcescens*, increased screening sensitivity, and reduce the potential for colony selection bias in future investigations, a novel PCR protocol was designed. The methodology of the final PCR protocol is detailed in Chapter 2.

Using the sample collection and processing methods developed during this pilot study, in combination with PCR testing and PFGE analysis, a larger environmental screening
investigation should be performed. Future investigations should include sampling from additional glasshouses in order to evaluate the distribution of the different *S. marcescens* strains within the facility, and to evaluate the relative importance of each potential environmental source of *S. marcescens* within the glasshouse environment. In addition to the sample types tested in the current study, mains water, plant material, potting soil and egg-laying substrate should also be investigated as potential environmental sources of *S. marcescens*. The question of whether *S. marcescens* type A1, the suspected outbreak strain in the insect colony, occurs in the environment requires further investigation. Sampling at different time points over a longer period would be necessary to discern any seasonal patterns in bacterial prevalence within the glasshouse environment.

The warm temperatures and high humidity conditions maintained within the glasshouses to mimic the climate on Lord Howe Island may be necessary for insect husbandry but could also favour the survival and growth of bacteria, such as *S. marcescens*, that thrive in moist environmental conditions (Yu, 1979; Miranda et al., 1996; Passaro et al., 1997; Jones et al., 2000; Brenner and Farmer, 2005). Regular disinfection of glasshouse surfaces, nest boxes and equipment, testing and filtration of water sources and protection of drinking water trays from contamination should be considered as precautionary measures to reduce the potential bacterial load in the environment and the likelihood of insects being exposed to high levels of environmental bacteria.

**6.5 CONCLUSION**

The environmental screening investigation was conducted as a small pilot study and a first attempt to locate potential sources of *S. marcescens* within the *D. australis* facility. As such, this investigation was not considered to be comprehensive for determining the distribution of *S. marcescens* in the glasshouse environment. Drinking water, frass, nest box surfaces and floor surfaces were all identified as potential environment sources of the bacteria. An environmental source of the previously identified outbreak strain, type A1, was not identified, however, two isolates representing the related type A2 strain were detected. A larger environmental screening study is required to further understand the distribution of *S.*
*marcescens* within the *D. australis* breeding facility, and to identify whether or not the suspected outbreak strain is present in the environment.
CHAPTER 7. EXPERIMENTAL INFECTION TRIAL

7.1 INTRODUCTION

To determine the pathogenicity of *S. marcescens* isolated from dead stick insects at post-mortem examination, an experimental infection trial was conducted. The experiment was performed with an aim to demonstrate the final two criteria of Koch’s postulates (Scott McVey and Czuprynski, 2013): that the isolated organism can produce the original disease when inoculated into a healthy, susceptible host, and that the same organism can be reisolated from the experimentally inoculated host.

In this experiment, 20 out of 40 apparently healthy, young adult, male stick insects were inoculated with the study isolate by oral administration. The insects were monitored over a period of 55 days, and a post-mortem examination was conducted for all insects at death, or at the completion of the study. Haemolymph from each insect was tested by culture and PCR to determine infection status. Histopathology was performed for a selection of PCR positive and negative insects. Insect frass was monitored by culture and PCR for the first 21 days of the trial. Finally, all isolates of *S. marcescens* cultured during the experiment were typed using PGFE analysis to determine their relatedness to the inoculating strain.

Ethics approval for this work was obtained through the Zoos Victoria Research and Animal Ethics Committee (reference: ZV15012).

7.2 METHODS

7.2.1 Study strain

Based on previous findings from the PFGE analysis of *S. marcescens* isolated at post-mortem examination (Chapter 5), the isolate AM923 was selected as the inoculating strain for the experimental infection trial. Isolate AM923 was representative of the Type A1 strain, the suspected outbreak strain in the current disease investigation. The isolate was originally isolated from an adult, male insect that was found dead in glasshouse 1.
7.2.2 Study location and duration
The experimental infection trial was carried out in glasshouse 1 (GH1) of the *D. australis* breeding facility at MZ during August to October 2015. This glasshouse was selected as it was not in use for housing any other stick insects at the time of the experiment. Insects were housed and treated within GH1 throughout the study period, and were monitored for a period of 56 days from the date of treatment.

7.2.3 Study subjects
Forty young adult, male stick insects were selected from two of the existing free-range populations of adult and sub-adult insects; 10 insects from glasshouse 6 (GH6) and 30 insects from glasshouse 7 (GH7). Each insect was examined by experienced invertebrate keepers to confirm that they had recently completed their final moult into adults, and appeared externally to be in healthy condition.

7.2.4 Study design
The 40 insects were divided into 8 groups of 5 insects. Each group was housed in a separate tall, rectangular mesh enclosure. A total of 20 insects were assigned to each of the ‘inoculated’ and ‘control’ treatment groups. The enclosures were spaced approximately 15 cm apart, and 15 cm away from the nearest wall. Each enclosure was numbered ‘E1’ to ‘E8’, based on its position within the glasshouse. The humidifier and fan were positioned on opposite ends of the same wall, near the ceiling. The layout of the glasshouse is shown in Figure 7.1b.

The 10 insects from GH6 were randomly divided between enclosures E1 and E8, while the 30 insects from GH7 were randomly divided between the remaining 6 enclosures: E2, E3, E4, E5, E6 and E7. The eight enclosures were allocated to alternating treatment groups, shown in Figure 7.1b. Enclosures E2, E4, E6 and E8 were allocated to the inoculated group, and enclosures E1, E3, E5 and E7 were allocated to the control group. For each enclosure, all five insects were subjected to the same treatment.
Figure 7.1. Diagrams of the set-up of (a) each mesh enclosure and (b) the glasshouse during the experimental infection trial. The enclosures allocated to the control group (E1, E3, E5 and E7) are shaded in blue and the enclosures allocated to the inoculated group (E2, E4, E6 and E8) are shaded in yellow.
7.2.5 Husbandry

The glasshouse was completely destocked of the previous stick insect occupants and emptied of equipment two weeks prior to the start of the trial. The mesh enclosures, nest boxes, water dishes and internal surfaces of the glasshouse were disinfected with bleach. The insects were moved into the glasshouse and placed into their respective enclosures 8 days prior to inoculation.

Within each enclosure, a wooden nest box with a removable lid, a shallow plastic drinking water dish, and a live feed plant in a glass vase were provided (Figure 7.1a). All enclosure groups were managed under identical husbandry conditions. Cuttings of tree lucerne (Chamaecytisus palmensis) were provided as feed, with fresh cuttings provided weekly, in excess of what the insects were expected to consume. Drinking water dishes and vases were refilled daily using mains water, but not cleaned or replaced during the course of the experiment. Frass was removed daily for 21 days, then every second day.

Humidity and temperature were regulated automatically within the target range of 22°C to 26°C, and 70 to 80% respectively, as per the conditions in the rest of the facility.

The glasshouse was kept under quarantine conditions during the entire study period, with access restricted to a few essential personnel and the mandatory use of disposable gloves and boot covers on entry. No equipment was shared with the other glasshouses. Insects were inspected and enclosures maintained daily for the duration of the trial.

7.2.6 Pre-infection screening

Water and pooled frass samples were collected from each enclosure 7 days prior to inoculation (Day -7) and again immediately prior to inoculation (Day 0) to screen for carriage of S. marcescens. Samples were screened by culture and PCR, with PCR performed as described in Chapter 2. For water, a sample (1.5 mL) was collected from each drinking water dish using a micropipette, at least 12 hours after the dishes were most recently filled. Frass samples were collected from the floor of each nest box, where most frass had been deposited by insects. Samples were collected into sterile microtubes.
Samples collected on each day were processed and cultured on LB/CHX agar as described in Chapter 6. For water samples, a 100 µL aliquot was plated directly, while frass samples were diluted to 0.01 mg/mL before a 100 µL was plated. All plates were incubated for 18 hours at 37°C. For each culture, up to 11 colonies suspected of being *S. marcescens* were selected based on morphological characteristics (see Chapter 3) and subcultured on DNase agar (Fort Richard Laboratories Ltd., Australia). The identification of the isolate was confirmed by PCR, where a fragment of the colony is used as a template.

Day 0 samples were additionally screened by plating dilutions directly onto DNase agar. A 100 µL aliquot of each diluted sample was spread on DNase agar and incubated for 18 hours at 37°C. DNase positive colonies were then tested by colony PCR.

A timeline of pre-infection screening, treatment, post-infection frass monitoring and euthanasia is shown in Figure 7.2.

![Timeline of pre-infection screening, treatment, post-infection frass monitoring and euthanasia in the experimental infection trial.](image)

**Figure 7.2.** Timeline of pre-infection screening, treatment, post-infection frass monitoring and euthanasia in the experimental infection trial.

### 7.2.7 Preparation of the inoculum and control

The inoculum was prepared by culturing *S. marcescens* AM923 in LB broth. A volume of 20 µL was selected as the inoculation dose. The methods for preparing the inoculum were based on preliminary growth curve experiments, in which the growth of AM923, cultured in LB broth at 37°C, was characterised over time using standard methods for serial optical density measurement and viable cell count determination (Talaro and Chess, 2012) (data not shown).
AM923 was inoculated on SBA from stock culture and incubated at 37°C for 24 hours. A single colony was selected using a sterile pipette tip and used to inoculate sterile LB broth (5 mL). The culture was incubated at 37°C for 18 hours with shaking. A log phase culture (10 mL) was prepared by incubation of $10^{-3}$ dilution of the culture in fresh LB broth for 4 hours with shaking. The two vials, one containing the inoculum (10 mL) and the other containing the control LB broth (100 mL), were kept on ice throughout treatment and transportation between the laboratory and the glasshouse.

The viable cell number of the inoculum was confirmed using standard viable cell counting techniques (Talaro and Chess, 2012) on LB agar and incubated at 37°C for 18 hours. An undiluted 100 µL aliquot of the negative control LB broth was also plated to check for contamination. This procedure was repeated for the inoculum and negative control, post inoculation, to confirm viable cell number and check for contamination.

### 7.2.8 Administration of treatment

The oral route of administration was selected to mimic the suspected natural route of transmission. On Day 0 of the trial, each insect was manually restrained and administered its designated treatment, using a micropipette. For the inoculated group, each insect was orally administered 20 µL of the inoculum ($2 \times 10^8$ cfu/mL). Each of the insects in the control group were administered 20 µL of sterile LB broth. Each dose of treatment was pipetted slowly and directly onto the insect’s mouthparts, to allow time for the insect to drink the liquid droplet. Once the entire droplet had disappeared into its mouthparts, the insect was returned to its enclosure. On average, less than 2 minutes was required to restrain and administer treatment to each insect.

Each vial of treatment was handled aseptically and each lid was replaced immediately between doses. A new sterile pipette tip was used for each insect. Clean, disposable nitrile gloves were worn at all times, and new gloves were worn for each enclosure to prevent cross-contamination. Treatments were performed in order of enclosure number, from E1 to E8.
7.2.9 Insect monitoring
Following treatment, insects were monitored daily for signs of illness, abnormal movement, abnormal behaviour, or death. At each inspection, each insect was manually handled briefly to allow visual inspection and assessment of its liveliness, responsiveness, coordination and strength. Any dead or severely ill insects were removed at this time. New nitrile gloves were worn for each enclosure, each day.

7.2.10 Frass monitoring
Between Day 1 and Day 20, a pooled frass sample from the floor of each nest box was collected and placed into a sterile microtubes. Samples were either processed for culture on the same day, or stored at -80°C for delayed processing. The remaining frass was removed from enclosures and discarded. A new pair of disposable gloves was used for handling each enclosure. After Day 20, frass was removed and discarded every second day. Frass samples were cultured for Days 1 to 9, and for Day 20 (Figure 7.2).

Each frass sample was processed as described in Chapter 6. A 100 µL aliquot of frass diluted at 0.01 mg/mL, was plated directly on DNase agar and incubated for 18 hours at 37°C. Samples collected on Days 1, 2 and 20 were processed for each individual enclosure, while samples from Days 2 to 9 were pooled for each treatment group during processing.

7.2.11 Euthanasia
Euthanasia was performed on all insects that remained alive at the end of the study period (n = 39), as described in Chapter 2. Insects from each enclosure were euthanised in a separate, large, plastic zip-lock bag.

To minimise the possibility of autolysis due to the time required to conduct the post-mortem examinations and sampling, insects were euthanised and processed over two days. All 20 insects from the control group were euthanised and processed on Day 54, while the remaining 19 insects from the inoculated group were euthanised and processed on Day 55 (Figure 7.2). All post-mortem examinations were completed between 35 minutes and 4 hours after euthanasia.
7.2.12 Post-mortem examination and sampling

Post-mortem examination was performed as described in Chapter 3. An individual name was assigned to each insect and its samples, corresponding to the enclosure in which it was housed, and the order in which the insects were processed within each enclosure group; i.e. insects and samples from enclosure 1 were named ‘E1-1’ to ‘E1-5’.

Two swab samples of haemolymph were collected through a unilateral cuticular incision. The first sample, for bacteriological culture, was placed into transport medium and stored at 4°C for 0-1 days before culture. The second sample, for PCR testing, was collected using a fine sterile swab and placed into 500 µL of RNAlater® stabilisation solution (Ambion®, Austin, Texas, USA). The sample in RNAlater® was stored at -80°C until DNA extraction.

Each insect was further dissected to examine the body for internal gross pathology. The foregut/midgut region was transected longitudinally into three tissue samples. The first sample was stored at -80°C, for culture if required. A second sample was fixed in 1:9 glutaraldehyde/paraformaldehyde solution (1 mL) for electron microscopy. The remaining sample was left attached to the gastrointestinal tract, which was fixed in 10% neutral buffered formalin, along with the rest of the body.

7.2.13 Haemolymph culture

Haemolymph swabs were cultured on SBA and DNase agar plates and incubated for 18 hours at 37°C. Culture plates were examined after 24 hours and 48 hours of incubation, where the morphology, density and DNase activity of colonies were recorded. Colonies with a non-pigmented, mucoid morphology on SBA and a DNase positive result, consistent with Serratia sp., were stored in 30% glycerol at -80°C. Colonies with a non-pigmented, mucoid morphology on SBA and an equivocal DNase result, were also stored for further testing by PCR.

7.2.14 PCR and DNA Sequencing

PCR targeting the haemolysin gene of Serratia sp., shlA, was performed to detect and confirm the identity of S. marcescens, following the protocol described in Chapter 2.
Colony PCR was performed for all suspect colonies cultured from frass, water, haemolymph and broth samples that were morphologically consistent with *S. marcescens* and positive for DNase activity. For PCR-positive colonies, the reactions were repeated with purified genomic DNA and the resultant PCR products were used as templates for DNA sequencing (Chapter 2). For the frass samples collected on Day 2, the PCR products generated from one colony of *S. marcescens* cultured from each enclosure were sequenced. For haemolymph samples stored in RNAlater®, DNA was extracted prior to PCR, following the methods described in Chapter 2, and the resultant products from the 14 PCR-positive samples with the strongest intensity bands were sequenced. Sequences were compared to that of the inoculating strain, AM923, using Geneious software (version R8.1.2) (Kearse et al., 2012).

7.2.15 Histopathology
The bodies of ten insects, including both PCR-positive and PCR negative insects, and at least one insect from each enclosure, were submitted for histopathological examination at a commercial laboratory (Gribbles Veterinary Pathology, Clayton, VIC). The insect from each enclosure with the most intensely positive PCR result band was included, and all of the insects with PCR-negative results (n = 2) were included.

7.2.16 PFGE Analysis
PFGE analysis was performed on isolates of *S. marcescens* cultured from haemolymph, water, frass and control broth samples. A dendrogram was then constructed to compare PFGE results to previous post-mortem haemolymph and environmental isolates. PFGE and dendrogram analyses were performed using the methods described in Chapter 2. For Day 2 frass isolates for enclosures E1, E2 and E8, image contrast was enhanced for the corresponding lanes prior to dendrogram construction. New isolates were typed according to the types assigned in the previous PFGE analyses (Chapters 5 and 6).

7.2.17 Statistical analysis
To compare the mortality counts, culture results, PCR results and histopathological results between the two treatment groups, a $2 \times 2$ contingency table was constructed for each treatment group (inoculated or control) against each outcome (positive or negative). For histopathology, only haemocoelomitis and enteritis lesions were considered for statistical
analysis. A Fisher’s exact test was performed for each variable using the statistical software, R (version 2.14.2) (R Core Team, 2016).

### 7.3 RESULTS

#### 7.3.1 Pre-infection screening
Pre-infection frass samples collected on both Day -7 and Day 0 tested negative for *S. marcescens* by selective DNase testing and colony PCR for all eight enclosures. However, when Day 0 frass samples were cultured directly on DNase agar, *S. marcescens* was detected for one enclosure (E8), demonstrated by positive DNase and PCR results.

Pre-infection water samples collected on Day -7 tested positive by selective DNase and by colony PCR for enclosures E1, E5 and E7. Water samples collected on Day 0 tested negative by selective DNase, PCR testing and direct DNase testing for all enclosures. DNase results were consistent with PCR results for all samples. All isolates of *S. marcescens* cultured were observed as non-pigmented.

#### 7.3.2 Calculated bacterial dose rate
The viable cell concentration of the inoculum was calculated to be $2 \times 10^8$ cfu/mL ($4 \times 10^6$ cfu/20 µL dose) prior to inoculation and at the completion of the experiment, 3.5 hours later. The inoculum was found to be pure culture at both time points.

No growth was observed for the control LB broth cultured at the start of the experiment, however, when the broth was cultured after the experiment, at 3.5 hours, it was found to be contaminated. Two colony types were found; medium-sized non-pigmented, mucoid colonies (220 cfu/mL), and tiny, translucent, white colonies (40 cfu/mL). The non-pigmented, mucoid colonies tested positive for *S. marcescens* by DNase and PCR testing. PCR sequencing results and PFGE results (Figure 7.5) found the contaminating isolate of *S. marcescens* to be indistinguishable from AM923, the inoculating strain. The maximum dose of *S. marcescens* that could have been administered to insects in the control group via the contaminated broth was calculated to be 4.4 cfu/20 µL dose, but it was not known when the contamination occurred.
7.3.3 Frass monitoring

*S. marcescens* was detected in pooled frass from both treatment groups by DNase testing and colony PCR from Days 1 to 9, except for Day 7. On Day 7, only the control group tested positive for *S. marcescens*. When enclosures were tested individually, *S. marcescens* was detected in frass from three enclosures (E1, E3 and E4) on Day 1 (post-infection), and in all eight enclosures on Day 2. On Day 20, frass tested positive for *S. marcescens* from all enclosures except for E6. All isolates of *S. marcescens* cultured were non-pigmented.

7.3.4 Mortality and morbidity

Over the 55 days of the study period, 1/40 insects died, while the remaining 39 insects survived until the end of the trial and were euthanised. Insect E8-1, from the inoculated group, presented dead in its nest box on the morning of Day 36, without any signs of illness detected prior to death. Since all insects had been confirmed as alive 15 hours earlier, it was concluded that the insect died less than 15 hour prior to its discovery and post-mortem examination. No further illness or death was observed in any of the insects during the remainder of the trial. The difference in the number of deaths in each treatment group was not found to be statistically significant (p = 1) using Fisher’s exact test.

Subjectively, all insects in the inoculated group were observed to show lethargy with slowed body and limb movements and reduced responsiveness on Days 1 and 2. However, these signs appeared to be self-limiting and gradually resolved by Day 7. The two treatment groups remained clinically indistinguishable for the remainder of the trial.

7.3.5 Post-mortem examination

The insect that died, E8-1, appeared moderately autolysed on post-mortem examination, with brown discolouration of the haemolymph and tissues, despite dying less than 15 hours prior to examination. No other gross lesions were detected. All of the remaining 39 insects were in good body condition with abundant, clear haemolymph, and no detectable gross lesions or discolouration.
7.3.6 Haemolymph culture and PCR

A heavy growth of *S. marcescens* was cultured in pure culture from the haemolymph of insect E8-1, confirmed by colony morphology, DNase activity and PCR. The isolate E8-1 was non-pigmented and non-haemolytic on SBA after 24 hours and 48 hours of incubation at 37°C.

No colonies consistent with *S. marcescens* were cultured from the haemolymph of the remaining 39 insects, based on observations of colonial morphology, DNase activity and results of the PCR. Haemolymph from insect E5-2 cultured a light, mixed growth of small non-pigmented and yellow colonies at 24 hours. The non-pigmented colony showed very slight DNase activity at 48 hours, but both colonies tested negative by colony PCR. Light to no growth was detected for 6 samples at 24 hrs and for a further 11 samples at 48 hours, however all colonies were negative for DNase activity. The difference in the number of culture positive and negative results in each treatment group was not found to be statistically significant (p = 1) using Fisher’s exact test.

The results of the PCR for the isolate E8-1 are shown in Figure 7.3. For E8-1, PCR products for both colony-extracted DNA and DNA extracted from haemolymph in RNAlater® were represented for comparison of band size. The PCR product for E8-1 appeared to be slightly larger than that for the inoculating strain AM923, with the expected product size of 473bp.

For haemolymph samples stored in RNAlater®, PCR positive results were demonstrated for 38/40 insects (Figure 7.4). For the PCR-positive insects, the intensity of bands approximately corresponding to a product of 473 bp varied greatly, and a variable number of additional faint bands corresponding to smaller products were also observed. Only samples from one insect from E3 (insect E3-4) and one insect from E4 (insect E5-3) demonstrated negative PCR results. Both PCR-negative insects belonged to the control group.

The total number of PCR positive results for each treatment group and each enclosure is summarised in Table 7.1. The difference in the number of PCR positive and negative results in each treatment group was not found to be statistically significant (p = 0.49) using Fisher’s exact test.
Figure 7.3. PCR results for the inoculating strain of *Serratia marcescens*, AM923, the isolate cultured from the haemolymph of insect E8-1, and DNA extracted from the haemolymph of insect E8-1. Lane 1: negative control (water only), Lane 2: positive control (AM923 DNA template), Lane 3: E8-1 (colony used as template) and Lane 4: E8-1 (purified DNA used as template). A 100bp molecular weight marker (M) is shown.
Figure 7.4. PCR results for DNA extracted from the haemolymph of each of the 40 insects in the experimental infection trial, a colony of inoculating strain AM923, and a colony of isolate E8-1. Insects are shown in order of enclosure number. The inoculated group insects are shaded in grey. Negative controls (N1, N2, N3) are shown. A 100bp molecular weight marker (M) is shown.
Table 7.1. Summary of PCR results for each treatment group and each enclosure in the experimental infection trial.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Inoculated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enclosure</td>
<td>E2</td>
<td>E4</td>
</tr>
<tr>
<td>Positive insects</td>
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</tr>
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<td>0</td>
</tr>
<tr>
<td>Total positives</td>
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<td></td>
</tr>
</tbody>
</table>

7.3.7 DNA sequencing

For the 14 haemolymph samples that were shown to have the most intensely positive PCR results (insects E1-1, E1-3, E1-5, E2-1, E2-4, E3-1, E4-2, E5-3, E5-5, E7-5, E8-1, E8-3, E8-4 and E8-5), sequencing of PCR product revealed sequences that were identical to that of the AM923, in all insects except for E8-1. The PCR product for E8-1 was shown to contain an additional sequence of 18 contiguous base pairs compared to AM923 and the other 13 PCR products. The unique 18 bp sequence was 5′GGGCCAGGACAAGGGTAA3′, occurring between positions 1473 and 1490 of the *shlA* sequence. The sequence of the *shlA* gene of *S. marcescens* cultured from all samples collected from insect E8-1 was found to have this difference.

For the *S. marcescens* isolates cultured from Day 2 frass samples from each enclosure, all 8 sequences were found to be identical to that of AM923.

7.3.8 Histopathological findings

The histopathological, culture and PCR results for the ten insects submitted for histopathology, including eight PCR-positive and two PCR-negative insects, are summarised in Table 7.2. All insects were found to have some lesions. The presence of haemocoelomitis lesions was strongly correlated to the presence of enteritis lesions. Considering only haemocoelomitis and enteritis lesions, the difference in the number of lesion positive and lesion negative results in each treatment group was not found to be statistically significant (p = 1) using Fisher’s exact test.
Table 7.2. Summary of histopathological results for ten stick insects from the experimental infection trial. Note: categories for which no specific comment was made by the pathologist are denoted by a dash (–).

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Insect name</th>
<th>Culture result</th>
<th>PCR result</th>
<th>Haemo-coelomitis</th>
<th>Enteritis</th>
<th>Gram stain</th>
<th>Hypercellular haemolymph</th>
<th>Other findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>E1-5</td>
<td>-ve</td>
<td>+ve</td>
<td>Mild</td>
<td>Mild</td>
<td>No bacteria seen.</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>E3-1</td>
<td>-ve</td>
<td>+ve</td>
<td>No</td>
<td>No</td>
<td>Rare Gram +ve coccobacilli.</td>
<td>Yes</td>
<td>Occasional cuticular erosions.</td>
</tr>
<tr>
<td>Control</td>
<td>E3-4</td>
<td>-ve</td>
<td>-ve</td>
<td>Mild</td>
<td>Mild</td>
<td>No bacteria seen.</td>
<td>Yes</td>
<td>Coccobacilli not associated with haemocyte aggregates. Occasional cuticular erosions.</td>
</tr>
<tr>
<td>Control</td>
<td>E5-4</td>
<td>-ve</td>
<td>-ve</td>
<td>Mild</td>
<td>Mild</td>
<td>Rare mixed bacteria on cuticular surface.</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>E5-5</td>
<td>-ve</td>
<td>+ve</td>
<td>Mild</td>
<td>Mild</td>
<td>Gram -ve bacilli and Gram +ve paired cocci.</td>
<td>Yes</td>
<td>Bacteria seen in haemolymph around gut, but rare elsewhere.</td>
</tr>
<tr>
<td>Control</td>
<td>E7-5</td>
<td>-ve</td>
<td>+ve</td>
<td>No</td>
<td>No</td>
<td>Occasional mixed bacteria.</td>
<td>Yes</td>
<td>Bacteria in shallow cuticular erosions.</td>
</tr>
<tr>
<td>Inoculated</td>
<td>E2-1</td>
<td>-ve</td>
<td>+ve</td>
<td>Mild</td>
<td>Mild</td>
<td>No bacteria seen.</td>
<td>Yes</td>
<td>Occasional cuticular erosions.</td>
</tr>
<tr>
<td>Inoculated</td>
<td>E4-2</td>
<td>-ve</td>
<td>+ve</td>
<td>Mild</td>
<td>Mild</td>
<td>No bacteria seen.</td>
<td>No</td>
<td>–</td>
</tr>
<tr>
<td>Inoculated</td>
<td>E6-5</td>
<td>-ve</td>
<td>+ve</td>
<td>No</td>
<td>No</td>
<td>No bacteria seen.</td>
<td>Yes</td>
<td>Occasional cuticular erosions.</td>
</tr>
<tr>
<td>Inoculated</td>
<td>E8-1</td>
<td>+ve</td>
<td>+ve</td>
<td>Moderate</td>
<td>Mild</td>
<td>Gram -ve bacilli.</td>
<td>Yes</td>
<td>Haemo-coelomitis lesions in head, limb and most severe in testis. Bacteria trapped in pigmented granulomas and dispersed throughout haemocoel.</td>
</tr>
</tbody>
</table>
7.3.9 PFGE results

Combined PFGE analysis results for cultured *S. marcescens* isolates are shown in Figure 7.5. The inoculating strain (AM923), control LB broth contaminant, pre-infection water isolates, and post-infection frass isolates for Days 1-3 are represented. Individual enclosure samples are represented for Days 1-2, while pooled group samples are represented for Days 2-3.

All isolates cultured from pre-infection water samples, post-infection frass samples from Days 1-2 and the control broth contaminant were represented by an identical band pattern to AM923. For Day 3 pooled frass samples, the isolate cultured from the inoculated group was also identical to AM923, but the isolate cultured from the control group differed from the previous isolates by at least five bands. The haemolymph isolate, E8-1, showed a distinctly different band pattern to any other isolate in the experiment, with a difference of at least eight bands from AM923.

The dendrogram constructed to compare the PFGE results of the experimental infection isolates of *S. marcescens* to the previous post-mortem haemolymph and environmental isolates is shown in Figure 7.6.
Figure 7.5. PFGE results for *Serratia marcescens* isolates cultured from pre-infection water samples, post-infection frass samples, the control broth contaminant, and haemolymph from insect E8-1, compared to the inoculating strain, AM923. Lanes have been rearranged by sample type and date of collection.
Figure 7.6. Dendrogram comparing PFGE results for *Serratia marcescens* isolates from the experimental infection trial (n = 19) to pure-culture post-mortem haemolymph isolates (n = 22), environmental screening isolates (n = 5) and control isolates VW347 and VW348 from the preliminary disease investigation (n = 2). The dendrogram was constructed using the Dice similarity method with UPGMA linkage and 3% tolerance. Coloured boxes indicate those isolates designated as type A1 (blue), type A2 (green), type B1 (orange) and type B2 (red). The three isolates shown in white were designated as type A, with no subtype.
7.4 DISCUSSION

No statistically significant differences in mortality count, culture results, PCR results or histopathological lesions were demonstrated between the inoculated group and the control group in the current study.

The isolation of *S. marcescens* type A1 from 3/8 water dish samples during pre-infection screening (Day -7) indicated that bacteria indistinguishable from the inoculating strain, based on the results of PFGE (Figure 7.6), were present in glasshouse 1 prior to experimental inoculation. This finding indicated either a contaminated water source, insects shedding *S. marcescens* due to pre-existing infections, or a ubiquitous presence of *S. marcescens* type A1 in the glasshouse environment. The isolate AM923, used as the inoculating strain, was originally isolated from an insect from the same glasshouse, which provided further evidence that the strain had been present within the glasshouse prior to experimental inoculation. However, *S. marcescens* was not detected when water dishes were retested immediately prior to inoculation (Day 0). The results of pre-infection frass screening were not suggestive of pre-existing gut colonisation, but low-grade colonisation, undetectable by culture, could not be ruled out. The presence of *S. marcescens* in the environment could have resulted in insect infections occurring independently of experimental inoculation. All three enclosures from which culture-positive water samples were collected (E1, E5, and E7) were intentionally assigned to the control group to avoid any confounding effects of the water-borne *S. marcescens* on the inoculated group.

Due to the contamination of the control broth with *S. marcescens*, no true negative control existed for this infection trial. The viable cell concentration of *S. marcescens* in the control broth at the end of treatments was calculated to be 220 cfu/mL (4.4 cfu/20 µL dose). Whether the broth became contaminated by the inoculum, or by an environmental source of *S. marcescens* is unclear. As the control broth was demonstrated to be sterile prior to inoculations, it was assumed that the contamination had to have occurred between the first and last treatments for the control group. Therefore, the actual dose of *S. marcescens* administered to each insect was likely to be between 0 and 4.4 cfu/insect. Since the inoculum
contained a $10^6$ times more concentrated dose than the control broth, it was assumed that some difference in infection outcome would still be observed between the treatment groups. However, the possibility that the contamination was sufficient to result in positive PCR results and histopathological lesions in the control group could not be ruled out. Individual dose vials of sterile control broth should be considered for future experiments to avoid contamination. Additionally, all insects in the control group should be treated before those in the inoculated group, to reduce the risk of contamination of the control group during treatments.

Culture results for frass samples indicated that *S. marcescens* was shed by insects in 8/8 enclosures from 2 days after treatment, and continued to be shed in both treatment groups for at least 20 days. This was the first time in the current study that *S. marcescens* was demonstrated in the frass of insects with known previous exposure. PCR, sequencing and PFGE results indicated that the isolates cultured from frass on Day 1 and Day 2 were indistinguishable from AM923. This suggests that AM923 had effectively colonised insects in inoculated group after treatment, but had also colonised insects in the control group. The detection of *S. marcescens* on Day 1 from only three enclosures (E1, E3 and E4) may have indicated an early phase of colonisation and shedding, or that the majority of samples collected that day represented gut contents prior to treatment. The isolate from the Day 3 pooled control group frass sample differed from AM923 by at least 5 bands, based on PFGE analysis, which indicated the presence of more than one strain of *S. marcescens* in the glasshouse.

Whether the control group insects had become colonised by the contaminated control broth, cross-contamination from the inoculated group, or an indistinguishable strain of environmental *S. marcescens* is unclear. Contaminated water dishes could explain the colonisation of insects in E1, E5 and E7, but not E3. Additionally, the colonisation of insects by an indistinguishable environmental strain at the same time as the experimental infection, but not prior, was considered unlikely.

The detection of *S. marcescens* in 7/8 enclosures on Day 20 was consistent with persistent gut colonisation and shedding, or colonisation of the nest boxes. Samples were not collected beyond Day 20 of this trial, as persistent bacterial shedding had not been anticipated.
Collection of frass over longer time period should be considered for future experiments to assess the maximum duration of shedding.

During the 55-day study period, 1/20 inoculated insects (5%) died, 0/20 control insects died, and no insects showed any signs of illness. The insect that died, E8-1, had been inoculated with AM923. However, the *S. marcescens* isolate that was cultured and detected by PCR from its haemolymph was distinct from AM923 and all other isolates, based on PFGE typing and DNA sequencing results. Sequencing results demonstrated that the PCR product for E8-1 (491 bp) differed from that of AM923 (473 bp), which may indicate a single insertion or deletion event. This 18 bp size difference was consistent with PCR electrophoresis results (Figure 7.3). The additional sequence in the haemolysin gene could possibly explain the lack of haemolytic activity observed when cultured on SBA. The role of haemolysis in pathogenicity in *D. australis* is uncertain.

Histopathologically, insect E8-1 was the most severely affected by haemocoelomitis of the 10 insects submitted. The histopathological findings, combined with culture and PCR results, suggested that the insect died due to a haemocoelomic infection by *S. marcescens*. It was unclear if the apparent autolysed condition of the insect at post-mortem examination was due to unusually rapid autolysis, or pathology due to the infectious disease process. PFGE analysis demonstrated a difference between *S. marcescens* isolated from E8-1 and AM923 of at least 8 bands, indicating that the 2 isolates were not closely related and the presence of E8-1 could not be explained by simple mutations from AM923 (Tenover et al., 1995). *S. marcescens* (E8-1) was grouped as a type A isolate at 87% similarity, but did not fall into either of the major subtypes or resemble any other isolate identified throughout the current study. This strain was not isolated from any frass sample or any other insect, suggesting that it may not have been shed into the environment at quantities detectable by culture, in contrast to AM923. It appeared that *S. marcescens* (E8-1) represented a rare, non-haemolytic and potentially pathogenic strain within the insect colony.

Positive PCR results for 38/40 haemolymph samples, compared to the culture results (1/40 positive) suggested that most of the insects had a low-grade haemocoelomic infection by *S. marcescens* that failed to be detected by culture methods. Since *S. marcescens* was observed
to grow well from haemolymph samples cultured on the same media (SBA and DNase agar) in previous experiments, inhibition of growth was not considered a likely explanation for negative culture results. Cross-contamination of PCR samples from the inoculated to control group was not considered likely since all control insects were sampled a day before the inoculated insects. PCR positive results were poorly associated with death, clinical illness or gross lesions. Mild to moderate histopathological lesions were reported in 6/8 PCR positive insects, but for insects that appeared to be infected with AM923 based on sequencing results, only mild haemocoelomitis was observed. However, the correlation between PCR results and histopathological lesions appeared poor, since both PCR negative insects also showed mild lesions. The presence of multiple product bands on PCR of DNA extracted from RNAlater® samples suggests that further optimisation of conditions of the PCR protocol is required before the assay can be widely used for detection of *S. marcescens*.

It is unclear if the PCR and histopathological findings indicate an early stage of disease progression, which may have eventually manifested as illness or death over a longer study period, or chronic low-grade infections with little clinical significance. Since the experiment was concluded at 55 days, it was not possible to exclude the possibility that clinical disease and deaths might have manifested over a longer timeframe, as a result of a chronic disease process. Pre-infection PCR testing of haemolymph was not performed due to the lack of a safe ante-mortem collection technique that would not inflict a breach of the exoskeleton. Further testing of haemolymph in healthy insects is required to determine the prevalence of insects with PCR positive haemolymph and the likelihood of pre-existing infections.

The enclosure E8 was associated with the most intensely positive bands on PCR results, corresponding to the most abundant PCR product from *shlA* amplification, compared to all other enclosures. E1 and E8 both housed insects sourced from GH6, in contrast to the other 6 enclosures, which housed insects from GH7. However, similarly intense PCR positive results were not demonstrated for the insects in E1. No evidence was found to suggest that the results of the trial were affected by the insects’ glasshouse of origin.

A possible explanation for the low number of deaths despite apparent colonisation by *S. marcescens* included the absence of a necessary environmental condition or stressor to
potentiate disease, such as unfavourable temperature, humidity or overcrowding. The set-up of the trial required each group of insects to be housed in a relatively confined space with only four other insects, in contrast to the regular adult/sub-adult glasshouses. This set-up may have inadvertently favoured survival or recovery from infection due to reduced competition for resources, and reduced effort required to access food and water. The absence of females may have also eliminated any physiological stress or competition due reproductive behaviours normally present in mixed sex adult groups. However, the daily physical handling of insects for monitoring was suspected to have exerted some degree of stress on the insects. Alternatively, AM923 may represent the most common strain of S. marcescens in the colony, but not necessarily a highly pathogenic strain, the dose administered may have been insufficient to cause disease, or the duration of the trial was not sufficient to observe disease due to this strain.

Since this was the first experimental infection study performed in D. australis, no reports of a suitable inoculating dose or duration existed in the current literature for this species. The dose was based on reported doses for oral inoculation of S. marcescens in other insect species (O’Callaghan et al., 1996; Connick et al., 2001). The dose volume was based the volume of sterile saline that could be administered practically to each adult insect by the oral route, validated in a small trial using an equivalent volume of sterile saline. Higher doses or a range of different doses should be considered for further infection investigations, which would be achieved either by extending the incubation time or concentration of a log phase culture. The 20 µL volume was appropriate for oral administration to adult insects, and a much larger volume is unlikely to be accepted. Smaller volumes would likely be required to inoculate younger insects.

Previous S. marcescens infection studies in other insect species have demonstrated much higher rates of mortality and much shorter inoculation-death intervals than observed in the current trial. In sugarcane borer larvae, 95% mortality occurred 5 days after inoculation by pin prick (King et al., 1975). In termites, 80% mortality occurred 6.7 days after inoculation via exposure to paper disc (Connick et al., 2001). In adult Lucilia sericata flies, between 10% and 45% mortality occurred 7 days after oral administration via capillary tubes (O’Callaghan et al., 1996). Finally, in adult boll weevils, 50% mortality occurred 48 hours after oral administration.
via cotton squares, and 69.9% mortality was observed after direct injection of 10 bacteria per insect (Slatten and Larson, 1967). The oral doses in the mentioned studies were comparable to the inoculum in the current trial but this was the first study, to this author’s knowledge, to attempt direct oral inoculation of insects using a micropipette. The oral route of inoculation was chosen for this experiment to mimic what was suspected to be the natural route of transmission for S. marcescens in the D. australis colony. Based on the previously mentioned insect studies, direct haemocoelomic injection may have resulted in a higher mortality rate and better demonstrated the potential pathogenicity of AM923, or lack thereof. However, haemocoelomic injection would not have accounted for the effects of the gut wall and normal host defences against bacterial invasion of the haemocoel. The possibility of alternative routes of infection were not investigated in the current study.

The effects of insect age and sex were excluded by selecting only young, adult, male insects in the current trial. Based on existing knowledge of the timing of expected moult ing stages (Carlile et al., 2009), all subjects were estimated to be 6-7 months old, and any insects nearing the end of their natural life expectancy (18 months) were excluded. More precise determination of individual insect age was not possible due to the lack of permanent individual identification techniques. Since the loss of healthy, young breeding females was considered to pose a negative impact on breeding objectives, only males could be included in trial. The effect of AM923 on females, juveniles and sub-adults has not yet been determined.

In an ideal experimental infection trial, insects should be reared under strict quarantine conditions, with continuous filtration and regular screening of water sources, regular screening of frass, and regular disinfection of equipment to minimise the possibility of pre-existing infections. Rearing insects from hatch would also allow precise age determination for each insect and may afford the use of female insects for study. However, significant resources and time would be required to rear an isolated population of D. australis from hatch to adulthood under quarantine conditions, and a large number of insects would need to be reared to account for natural attrition. Additionally, potential environmental sources of S. marcescens, such as live plant material and natural substrate, may be unavoidable.
Due to the difficulty of using valuable individuals of a highly-endangered species for infection studies that end in death or euthanasia, it may be preferable to perform additional experiments on a more common stick insect species. Using a different species as a model may introduce uncertainty about the applicability of results to *D. australis*, but would potentially allow a much larger insect sample size, including different ages and sexes.

In summary, high virulence of *S. marcescens* AM923 was not demonstrated in male, young adult *D. australis* after direct oral inoculation of $4 \times 10^6$ cfu/insect, under the current experimental conditions. Low-level infection of the haemocoel, mild histopathological changes and persistent faecal shedding were demonstrated in association with AM923, but no mortalities could be attributed to the strain. At present, AM923 and *S. marcescens* type A1 should be considered as a possible pathogen, with low virulence under the current experimental conditions.
CHAPTER 8. GENERAL DISCUSSION

8.1 OVERVIEW OF MAJOR FINDINGS

*S. marcescens* was frequently isolated from the haemolymph of dead and moribund individuals of *D. australis* within the captive breeding colony at Melbourne Zoo. Widespread infectious disease has important implications not only for the health and management of the breeding colony, but also the prospect of future reintroduction and long-term survival of *D. australis* in the wild.

Multiple strains of *S. marcescens* were detected in the haemolymph and frass of insects, and in the glasshouse environment. Drinking water, frass, floor surfaces and nest boxes were identified as potential reservoirs within the glasshouse environment. Type A1 *S. marcescens* was implicated as the most likely outbreak strain, based on PFGE analysis, and was frequently isolated from the haemolymph of dead insects but not from the environment. Type A2 *S. marcescens* was also frequently isolated from dead insects, and also from the environment. The type A2 strain is suggested as a possible epidemiological origin of the more insect-associated type A1 strain. Type B strains appeared to have a limited role in insect disease.

The virulence of the *S. marcescens* type A1 strain appeared to be limited under experimental conditions but low-level haemocoelomic infections and persistent faecal shedding of the organism were common. *S. marcescens* should be considered as a potential pathogen of *D. australis*, associated with haemocoelomitis and enteritis. It most likely represents an opportunistic pathogen, causing disease under specific environmental or host conditions. This was consistent with previous research on the relationship between *S. marcescens* and other insect species (Steinhaus, 1959; Grimont and Grimont, 1978; Dillon and Dillon, 2004; Mohan et al., 2011). Increased mortality rates were found to be strongly associated with the warmest months of the year, and high local temperatures. High stocking density of glasshouses were not identified as a risk factor for increased mortality rate.
Early detection of disease in *D. australis* is currently hindered by: the subtleness of physical and behavioural changes, the lack of measurable and objective parameters to assess health, the lack of safe ante-mortem testing methods, and the typical motionless posture displayed by even normal stick insects. Additionally, the nocturnal lifestyle of adult insects results in limited opportunities to observe them when they are typically active and outside of their nest boxes. Due to the cryptic nature of disease and behaviour in *D. australis*, discrimination between healthy and diseased insects presents an ongoing challenge. The majority of insects present acutely dead, or in a moribund state, without any preceding signs of illness. Thus, whether ‘sudden death’ events truly represent peracute disease, rather than a chronic disease process that is only detectable in the late stages, remains unclear.

During the current study, attributing insect death to a definitive cause was not often possible and juvenile insect mortalities were under-represented due to the challenges in finding their bodies. Due to these reasons, the mortalities included in this study represent all adult deaths and a small subset of juvenile deaths, regardless of the cause of death. Additionally, due to the absence of distinguishing physical features or permanent individual identification markers, precise age determination was not possible, and all insect ages were approximated based on size and exoskeleton appearance. These limitations should be taken into consideration when interpreting mortality and infection rate findings.

Screening of haemolymph and frass from apparently healthy insects by PCR and culture is recommended to better understand the prevalence and clinical implications of *S. marcescens* infections in the *D. australis* colony. Widespread, low-level positive results may suggest that *S. marcescens* is either an endemic pathogen in the colony, or that it represents a normal commensal organism in low numbers. Alternatively, rare positives may provide evidence that any *S. marcescens* colonisation is an abnormal process. Since the current PCR test targets a highly-conserved region of the *shlA* gene, a diverse range of *S. marcescens* strains can be detected using this test. Genome sequencing and design of additional PCR primers targeting more variable gene regions are required to enable detection of specific strains of importance in the colony. The question of whether the shedding of *S. marcescens* in frass represents...
infectious disease or normal colonisation by gut flora needs to be addressed before frass screening could be used as a meaningful diagnostic test for live insects.

Since *D. australis* remains critically endangered in the wild, and safe ante-mortem haemolymph sampling methods have not yet been established, much of the bacteriological work in the current study was performed on dead and euthanised insects. Exclusive sampling of dead and moribund insects may heavily skew culture results to represent a high proportion of diseased individuals, or those that have undergone some degree of post-mortem autolysis. In order to determine the relevance of research findings to live insects, and to establish normal parameters for healthy insects, testing of healthy insects may be unavoidable.

Testing healthy insects would require either the development of a safe method for ante-mortem haemolymph sampling, or euthanasia of healthy insects. The difficulty of distinguishing apparently healthy and subclinically diseased insects would remain a complicating factor in a live insect investigation. Histopathology appears to be a good, accessible method for detecting microscopic disease, but can only be performed if insects are dead or euthanised. If insects are euthanised for research, the number of individuals used would need to be balanced against the overall conservation goals of the breeding program, the insect numbers required to maintain a stable population, and the required statistical power of the investigation. While sacrificing healthy individuals of a critically endangered species would seem counterintuitive for a breeding program aimed at conserving the species, it could also be argued that safe reintroduction of insects into the wild is not possible without first establishing what is normal for the species.

Using the culture and PCR methods developed in the current study, additional testing of potential environmental sources, including water, frass, plant material and soil, is recommended to gain a better understanding of the distribution and prevalence of *S. marcescens* strains within each glasshouse, and the relative importance of each environmental source. Since *S. marcescens* appears to represent an opportunistic pathogen that is present in both insects and the environment, destocking or test and cull methods cannot be recommended as appropriate or effective disease control measures for the colony.
Culling insects with *S. marcescens* may result in a substantial decline of insect numbers with little effect on the prevalence of the bacteria.

Preventative measures to reduce the risk of bacterial contamination of drinking water and food are recommended. Water dishes placed on the ground, near nest box stations are prone to contamination by falling frass and plant material or by insects walking through the water. A modified design similar to commercially-available poultry drinkers, with an additional cover, is suggested as a simple and inexpensive solution to protect water dishes from falling material and insect entry. Drinkers should also be placed away from any overhanging structures to further reduce the risk of contamination. The current nest boxes are prone to accumulation of frass material, which can cause insects to nest in close proximity to frass and potential faecal microbes. A nest box design that allows frass to fall into a removable collecting tray is recommended to reduce faecal exposure.

The correlation between mortality rate and temperature suggests that additional measures to prevent extreme heat during hot weather, including active cooling, increasing ventilation, or insulating glasshouses from external temperatures, may lead to reductions in mortality rate. Analysis of historical records of internal glasshouse temperature and humidity data against mortality rate data, is recommended to further assess environmental risk factors and identify periods of extreme fluctuations. Regular analysis of mortality rate and husbandry data, including temperature, humidity, egg hatch rates and population size, may facilitate the identification of specific risk factors for each glasshouse, early recognition of abnormal trends, and early intervention to prevent or mitigate emerging problems.

The effects of the extreme genetic bottleneck and suspected low genetic diversity in the surviving wild and captive *D. australis* populations remains an area of research that requires attention. Genetic studies of *D. australis* have not been conducted to date, but are expected to become a focus of research in the near future. Until such information is available, the hypothesis of impaired insect immunity due to limited genetic diversity remains unconfirmed.

Since *S. marcescens* is a potential zoonotic pathogen (Miranda et al., 1996; Hejazi and Falkiner, 1997; Grimont and Grimont, 2006), personnel and visitors who engage in insect
handling or husbandry procedure should wear gloves during such activities, and should wash hands thoroughly before and after handling insects to prevent the transmission of infectious agents between insects and humans. Appropriate uniform policies for keepers should be enforced to minimise the risk of exposing family members and pets to bacteria via contaminated clothing or footwear. To the author’s knowledge, incidents of acquired human infection with *S. marcescens* from the colony have not been reported.

8.3 IMPLICATIONS FOR REINTRODUCTION AND THE FUTURE OF *D. australis*

At present, the introduced black rat on Lord Howe Island continues to present a great threat to the prospect of successful reintroduction and survival of *D. australis* to the island (Wilkinson and Priddel, 2011). Until the rat is successfully eradicated from the island, *D. australis* reintroduction cannot be considered, and the Melbourne Zoo breeding colony remains crucial to maintaining a viable population of healthy insects for future reintroduction. The implications of *S. marcescens* to the prospect of successful *D. australis* reintroduction on Lord Howe Island currently remain unclear. Additionally, the risk of the current strains of *S. marcescens* to humans and native animals on Lord Howe Island remains unknown.

Testing frass and soil samples from the colony of *D. australis* on Ball’s Pyramid, and from areas of likely habitat on Lord Howe Island, may provide further insight into the significance of *S. marcescens* in *D. australis*. If *S. marcescens* related to the current strains can be demonstrated in samples from those locations, the likelihood of the bacteria being a novel disease threat would seem low. If that is true, insects would not need to be free of *S. marcescens* to be considered safe for reintroduction. However, if the *S. marcescens* is absent from Ball’s Pyramid and Lord Howe Island samples, vigorous screening of insects would be recommended for all insects considered for release, to identify infected and carrier insects.

The potential stresses of long-distance relocation and acclimatisation to new environmental conditions may be a compounding risk factor for disease in reintroduced insects. Other than during the initial relocation of a small number of insects between Ball’s Pyramid, New South Wales and Victoria in 2003 (Carlile et al., 2009), long-distance relocation has not previously been observed for the species. Additionally, due to indoor housing, the captive population
has never been exposed to normal weather phenomenon such as rain, wind chill, storms or direct sunlight. Whether those environmental conditions would affect insect disease susceptibility, morbidity and mortality in the wild is not yet known.

The transportation of insect eggs could provide an alternative solution to transporting live insects that circumvents the potential issues of stress and acclimatisation, while increasing the opportunity for screening. While vertical transmission of *S. marcescens* via colonised eggs has been demonstrated in other insect species (Bell, 1969; Sikorowski and Lawrence, 1998; Inglis and Lawrence, 2001), egg colonisation in *D. australis* has not yet been investigated in detail and requires further work. Egg batches should be screened for *S. marcescens* by culture and/or PCR prior to transportation. Since egg numbers are typically numerous, a proportion of eggs from each batch could be randomly selected and sacrificed for testing without significant impact on the insect population. For egg transportation to be a viable solution for species reintroduction, an insect hatchery and rearing facility on Lord Howe Island would be required.

The findings from this current study suggest that the presence of *S. marcescens* does not present a major threat to the captive breeding colony of *D. australis*, despite its potential to cause disease in some individual insects. However, uncertainty remains surrounding the implications of *S. marcescens* in individuals that are eventually released to the wild, and to the health of the future wild population. Further research is required to ensure that the role of *S. marcescens* in disease of *D. australis* is fully understood before efforts to reintroduce the insect to the wild can commence.
APPENDIX 1

SUPPORTING INFORMATION FOR CHAPTER 7

The growth kinetics of *S. marcescens* isolate AM923 were characterised in two simple growth curve experiments, based on serial measurements of optical density (OD) and colony forming unit (cfu) over time, in order to allow prediction of the concentration and number of viable bacterial colony forming units (cfu) that would be administered to each insect in the experimental infection trial (Chapter 7). Based on the results of these experiments, an ideal dilution factor and incubation time for the inoculum were determined. The viable bacterial concentration (cfu/mL) of the inoculum and the dose rate of viable bacteria per insect (cfu/20 µL) were predicted.

In each experiment, AM923 was cultured aerobically on SBA at 37°C for 24 hours, then a single colony was suspended in sterile LB broth (5 mL). The inoculated LB broth was incubated at 37°C for a further 18 hours, before the culture was serially diluted 1:9 in sterile LB broth, to eight dilution factors between $10^0$ and $10^{-7}$. The final diluted cultures were incubated aerobically at 37°C, in a Nuclon™ Delta Surface 96-well plate (Thermo Scientific™, Roskilde, Denmark). OD was measured using a Synergy™ H1 OD reader (BioTek®, Winooski, Vermont, USA). For each dilution, serial OD measurements were performed hourly, commencing immediately after dilution. Mean OD measurements were calculated based on the OD readings from three wells for each dilution.

In the first experiment, OD measurements but not cfu counts were not performed, hourly for 12 hours, then 6-hourly for a further 18 hours. In the second experiment, both OD measurements and cfu counts were performed hourly for 6 hours, for only the $10^{-3}$ dilution. cfu concentration (cfu/mL) and log cfu concentration ($\log_{10}(\text{cfu/mL})$). Growth curves were plotted from the results of the two experiments, shown in Figure A1.1 and Figure A1.2 respectively.
Figure A1.1. Mean optical density measurements between 0 and 30 hours for *S. marcescens* isolate AM923, cultured in LB broth at eight serial dilutions between $10^0$ and $10^{-7}$. Each culture was incubated aerobically at 37°C for 30 hours.

Figure 7.2. Mean optical density measurements and $\log_{10}$ cfu concentration ($\log_{10}(\text{cfu/mL})$) between 0 and 6 hours for *S. marcescens* isolate AM923, cultured in LB broth at a $10^{-3}$ dilution. A linear fit line for the $\log_{10}(\text{cfu/mL})$ data is shown as a blue dashed line.
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Title:
A bacteriological disease investigation in a captive breeding colony of Lord Howe Island Stick insects (Dryococelus australis)

Date:
2017

Persistent Link:
http://hdl.handle.net/11343/194101

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