Diversity of protozoan parasites in a threatened marsupial (*Petrogale penicillata*) which is part of a conservation program

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This thesis is presented for the degree of Doctor of Philosophy

August 2016

Declaration

The research described in this thesis is original and has not been submitted, in any form, for the award of a higher degree at another university or tertiary institution. I consent to a copy of this thesis being made available through Macquarie University library for consultation, loan and photocopying, subject to the provisions of the Copyright Act 1968.

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Date

Abstract

In conservation management, parasite communities are often only considered from a disease perspective, yet parasites are an important part of biodiversity. The role that parasites fulfil in conservation management is largely unknown. Hence, this research examines the biodiversity and prevalence of three protozoan parasites of the threatened brush-tailed rock-wallaby (BTRW, *Petrogale penicillata*) and more broadly, parasite diversity and specificity in the genus *Petrogale*, which contains seven species of conservation concern.

The parasites of the seventeen *Petrogale* species are diverse with 157 species recorded across the genus. Parasite assemblages of *Petrogale* were found to have a significant relationship with the phylogeny (p = 0.008) and biogeography (p = 0.0001) of their hosts, and thus endemic parasites in threatened populations may be at risk of co-extinction.

Faecal samples from brush-tailed rock-wallabies (BTRW) which were part of a conservation program were collected from nine sites under three categories: three sites with captive bred animals, four sites supplemented with captive bred animals and two sites with purely wild animals. *Cryptosporidium sp*. and *Giardia sp*. had a low prevalence across all BTRW sites, 7.1% (23/324) and 6.3% (20/318) respectively. No significant differences were observed in prevalence between site categories. Conversely, *Eimeria* was ubiquitous across all site categories, with an overall prevalence of 92.3% (108/117). The infection intensity (oocysts/gram faeces) of *Eimeria* did not vary significantly between site categories, but the highest variation was observed in supplemented sites.

Cryptosporidium positive samples from two captive sites and one wild site contained the marsupial-specific *C. fayeri* and one captive and four wild samples were identified as the broader host range *C. meleagridis* using multi-locus genotyping (18S rRNA, actin and *gp60* loci). Similarly, the anthropozoonotic *G. duodenalis* assemblage A was identified in eight

supplemented, two captive and one wild sample, and assemblage B in seven supplemented and four wild samples using the 18S rRNA, β-giardin and *gdh* loci. The occurrence of parasite species associated with humans and domestic animals in BTRW indicates that these parasites are being transmitted via the environment as a result of contamination from non-wallaby sources.

Next-generation sequencing (NGS) was used to examine the genetic structure of *Eimeria* communities. A new methodology incorporating a partial fragment of the 18S rRNA locus (450 bp) was developed to enable community analysis of *Eimeria*. Analyses revealed 28 *Eimeria* operational taxonomic units (OTUs) in 53 BTRW samples. This high biodiversity of *Eimeria* was maintained across all site categories with no significant difference in OTU richness or composition between sites or site categories. A high overlap in phylogeny and OTU composition was observed between captive bred and supplemented sites, suggesting geographical isolation of the captive bred populations may alter their *Eimeria* communities but further research is required to understand this variation.

While no impact of the conservation program was detected on the BTRW parasites, anthropozoonotic parasites were present in both wild and captive BTRWs and thus present in their natural environment. As parasites of Australian wildlife are diverse and endemic, the parasite communities of endangered species need to be monitored, using similar methodology as this study, and considered within conservation management.

Chapter Description

Chapter 1: General introduction, research objectives and positioning of chapters

I reviewed the literature and wrote the chapter. Feedback was provided by my primary supervisor, Michelle Power and my co-supervisor, Mark Eldridge.

Chapter 2: Biodiversity and host specificity of parasites in the genus *Petrogale*: implications for conservation management.

I researched all published records of parasites in *Petrogale* with Ian Beveridge and Mark Eldridge checking the data. I wrote the literature review. I worked with David Nipperess to create the host specificity graphs and received feedback from him on this section. Further direction and feedback were provided by my supervisor Michelle Power, my co-supervisor Mark Eldridge and co-author Ian Beveridge.

Chapter 3: Diversity of *Cryptosporidium* in brush-tailed rock-wallabies (*Petrogale penicillata*) managed within a species recovery program

Faecal samples were collected opportunistically from brush-tailed rock-wallaby (BTRW) populations by volunteers from the New South Wales (NSW) Office of Environment and Heritage, organised by Deborah Ashworth and Todd Soderquist. DNA extractions from faecal samples were performed by myself and our research assistant, Lachlan Byatt. I conducted all polymerase chain reaction (PCR) lab work, including optimising the PCR for *Cryptosporidium* in BTRWs with advice from my supervisor, Michelle Power. I carried out all the screening at the 18S rRNA locus and subsequent amplification at other loci. Sequencing was performed by Macrogen (Seoul, South Korea), and I analysed the sequences. I conducted the statistical work with advice from Koa Webster. The paper was written by me

with direction by my supervisor Michelle Power, co-supervisor Mark Eldridge and co-author Deborah Ashworth.

Chapter 4: Investigation into potential transmission sources of *Giardia duodenalis* in a threatened marsupial (*Petrogale penicillata*).

Faecal samples were obtained from the NSW Office for Environment and Heritage (same samples as used in Chapter 3) and DNA was extracted by myself and Lachlan Byatt. I optimised the PCR for *Giardia duodenalis* with advice from Michelle Power and Amy Asher. I performed all PCRs, sequencing was performed at Macrogen and I analysed the sequences. I undertook the statistical analysis advised by Koa Webster. I wrote the paper, with feedback from my supervisor Michelle Power and co-supervisor Mark Eldridge.

Chapter 5: Evaluation of next generation sequencing for the analysis of *Eimeria* communities in wildlife

Faecal samples were obtained from NSW Office for Environment and Heritage (same samples as used in Chapters 3 and 4). A subset of samples was selected, with input from my supervisors based on quantity (~0.5 g required) and even representation across sites. I performed all zinc sulphate flotations and oocyst counts. I optimised the sucrose flotation technique for a smaller volume of faeces (0.5 g) and performed all flotations and DNA extractions. I designed the primers and optimised the amplification PCR, then performed all amplification PCRs. Preparation of the Nextera XT Index genomic libraries and sequencing on the Illumina MiSeq platform was performed by the Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Sydney, Australia. Data analysis and statistical analysis of the next-generation sequencing was performed by me with input and advice from

Matthew Lott. I wrote the chapter with feedback from my supervisor Michelle Power, co-supervisor Mark Eldridge and co-author Matthew Lott.

Chapter 6: Parasites on the hop: captive breeding maintains *Eimeria* communities in an endangered marsupial

This chapter utilised the data from Chapter 5 and applied it to the *Eimeria* communities from different sites and population categories of BTRW. Data analysis and statistical analysis was performed by me with aid and input from Matthew Lott and Koa Webster. I wrote the chapter with feedback from my supervisor Michelle Power and co-supervisor Mark Eldridge.

Chapter 7: General discussion and conclusions

In this chapter, I summarise the key points of my thesis, discussion their implications, and provide advice for future research directions. I wrote the discussion with feedback from my supervisor Michelle Power and co-supervisor Mark Eldridge.

Publications

Vermeulen ET, Power ML, Nipperess D, Beveridge I, Eldridge MDB (2016) Biodiversity of parasite assemblages in the genus *Petrogale* and its relation to the phylogeny and biogeography of their hosts. *Australian Journal of Zoology*, 64 (1), 61-80. DOI: 10.1071/ZO16023. Chapter 2.

Vermeulen ET, Ashworth DL, Eldridge MDB, Power ML (2015) Diversity of *Cryptosporidium* in brush-tailed rock-wallabies (*Petrogale penicillata*) managed within a species recovery programme. *International Journal for Parasitology: Parasites and Wildlife*, 4**,** 190-196. DOI: 10.1016/j.ijppaw.2015.02.005. Chapter 3.

Vermeulen ET, Ashworth DL, Eldridge MDB, Power ML (2015) Investigation into potential transmission sources of *Giardia duodenalis* in a threatened marsupial (*Petrogale penicillata*). Infection, Genetics and Evolution, 33**,** 277–280. DOI: 10.1016/j.meegid.2015.05.015. Chapter 4.

Vermeulen ET, Lott MJ, Eldridge MDB, Power ML (2016) Evaluation of next generation sequencing for the analysis of *Eimeria* communities in wildlife. *Journal of Microbiological Methods*, 124, 1-9. DOI: 10.1016/j.mimet.2016.02.018. Chapter 5.

Vermeulen ET, Lott MJ, Eldridge MDB, Power ML (2016) Parasites on the hop: captive breeding maintains biodiversity of *Eimeria* communities in an endangered marsupial. *Biological Conservation*, 200, 17-25. DOI: 10.1016/j.biocon.2016.05.019. Chapter 6.

Conference Presentations

Vermeulen ET, Eldridge MDB, Power ML (2013) The influence of conservation management on host-parasite relationships in brush-tailed rock-wallabies (*Petrogale penicillata*), Wildlife Disease Association – Australasia conference Grampians Victoria, 29 September – 4 October. Oral presentation.

Vermeulen ET, Eldridge MDB, Power ML (2014) Prevalence and diversity of *Cryptosporidium* in endangered brush-tailed rock-wallabies (*Petrogale penicillata*), Australian Society for Parasitology 50th Anniversary Annual Conference, Canberra Australia, 30 June – 3 July. Poster Presentation

*Received ASP Student Travel Grant

Vermeulen ET, Eldridge MDB, Power ML (2015) Diversity of *Cryptosporidium* and *Giardia duodenalis* in threatened brush-tailed rock-wallabies (*Petrogale penicillata*). New Zealand Society for Parasitology and Australian Society for Parasitology Annual Conference, Auckland New Zealand, 29 June – 2 July. Oral presentation.

*Received ASP Student Travel Grant

Acknowledgements

I went through some tough times prior to starting my candidature and so it is no small wonder and thanks to a lot of people that I made the crazy and stubborn decision to see a PhD through all the way. It has been a long road leading up to it, not to mention during the actual candidature and I want to highlight the people that helped me along that road.

First and foremost, I want to thank my principal supervisor, Michelle Power, for taking the chance with me and offering me this project. You have taught me a lot about the scientific method and scientific writing during the course of my candidature. Your input and advice has been invaluable during data collection, optimising methods and writing up the manuscripts. You have given me a great opportunity with this project and I will always be grateful for that. I would also like to thank my ever-chipper co-supervisor Mark Eldridge, who had equally valuable input in the direction of the project and advice on writing my manuscripts. I also enjoyed seeing the collections at the museum courtesy of you which allowed me the first glimpse of my study subject, even if the poor wallaby was stuffed in a Superman position.

Part of what made me enjoy my time over these three years was my great lab group. First of all, I wish to thank Lachlan Byatt, our research assistant and overall lab wizard who taught me a lot of the techniques and tools in the lab, as well as our many conversations in the office about whatever came to mind. I also learned a lot from my dear grad student colleagues, Matthew Lott and Amy Asher, about poo shakes, I mean, faecal flotations, wrestling with Giardia and how to make sense from strings of seemingly random combinations of the letters A, T, C and G. I thank Koa Webster for being a pillar of support and giving great advice on statistical analysis and proof-reading some of my writing, as well as chats about stuff, mainly Doctor Who. I would also like to thank everyone else that has come and gone through our lab during my candidature, with whom I also had a great time, commiseration and great chats,

sometimes about how to appease the PCR gods. So I would like to thank Sabine Schiller, Tina Wunderlin, Liana Wait, Sinead, Lucas, Daniel, and others that seemed to pop into existence around here. You all made my time here fun, so thank you very much and I hope we can still see each other in the future.

I would also like to thank other friends I made here while at the Department or while tutoring, too many to name but a few I would like to give honourable mentions are Silvia, Luke, Lara, Giselle, Winnie, Sarah, and so many others. On that note, I would also like to thank the Department of Biological Sciences in general for creating a great environment for grad students, and as always, thank you very much to the technical and administrative staff who made things run mostly smoothly with the scary admin stuff. The departmental drinks, morning teas, seminars, workshops and Christmas parties were always stuff to look forward to.

I would also like to greatly thank my co-author Ian Beveridge, one of the greatest parasitologists and kindest persons I've ever met. His insights into the worlds of worms inside other beings were invaluable and I learned a lot from working with him. I also thank my other co-author, David Nipperess, for aiding me in the use of R modelling for the review, which made things a lot spiffier. To Deborah Ashworth, Todd Soderquist and all the volunteers with National Parks/Office for the Environment and Heritage, thank you for all your wonderful samples. From wallabies anyway. Admittedly, I've never seen anyone get as excited about getting poop in the mail as my supervisor.

An important thank you goes to the support of my family, my parents, my sister and brother, the webcam-mediated support of my family in Belgium and my extended family in the Sieviers, even if most of the time they couldn't really understand the stuff that came out of my mouth when I tried to explain my work. Thanks for smiling and nodding, and in providing

unrelenting support whenever I needed it. And even though they can't read, I want to give a thank you to my dogs Ziva and Spyro, for always knowing what to do to cheer me up. You know, by being dogs.

A special thank you goes to my best friend, Amber Fowler. You may be far away in geography but you are ever close to me in my heart.

Most importantly, I want to thank my partner in life, love, crime and puns, Raymond Sievier. You stuck with me through thick and thin, for better or for worse, although we haven't said those words officially in front of everyone else yet, I'm sure that when it happens, puns will be involved. I would not be erroneous in stating that without you, I would never have made it this far. You always know what to say or do when I'm riding my personal emotional rollercoaster and you're my 'rots in the branding', my shore in a stormy sea, or whatever the saying in English really is. With you, I shall know no fear. So, with all my heart, thank you.

Lastly, I really, really wanted to add this great little line from one of my greatest inspirations for science, the ever great Carl Sagan, somewhere in my thesis so here goes:

Science as a candle in the dark

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1. General Introduction

1.1 Overview of conservation management

Global biodiversity is experiencing increased threats from processes such as loss of habitat, climate change and presence of invasive species (NRMMC, 2010). Conservation management aims to abate these threats by restoring biodiversity or increasing diminished populations of threatened species (Meffe *et al*., 2006). As reduced populations are at risk of inbreeding, maintaining and increasing genetic diversity is important to the overall population health (Browning *et al*., 2001). To re-establish gene flow and increase genetic diversity in fragmented, remnant populations, wildlife conservation managers employ strategies such as captive breeding and translocation of animals (Griffith *et al*., 1989, Hague & Routman, 2015). Captive breeding and translocating animals into new environments, however, carry risk factors that can potentially impact their health (Woodford & Rossiter, 1993, Cunningham, 1996, Moir *et al*., 2012). These risk factors include:

1. Atypical interactions with other animals (including humans) can introduce novel pathogens (Cunningham, 1996);
2. translocated animals can alter the host-parasite relationship of sympatric animals in the new environment, both released and local individuals (Arneberg *et al*., 1998);
3. stress through handling and introduction to a new environment can induce stress hormones and lower the immune system, thus exacerbating existing infections and making animals more susceptible to novel infections (Wynd *et al*., 2006, Tuft *et al*., 2011);

4. the use of anti-parasite drugs such as anticoccidials or anthelmintics can lower the infection intensity of the target parasite but may increase infection of non-target parasites (Knowles *et al*., 2013); and

5. in the captive breeding process, a species may be kept and bred in captivity over several generations (Schultz *et al*., 2006). During this time, the isolation from their native environment and their con-specifics and thus the absence of their natural parasites (Thompson 1999), this captive population may lose its immunity or resistance to these parasites and become immunologically naïve. When a generation is reintroduced into the wild, they may thus be susceptible to resident parasites in their habitat.

Parasites are often only considered in conservation from a disease management perspective due to the potential for introduction of novel parasites into naive populations (Horan & Melstrom, 2011). Viewing parasites only from a disease perspective is likely due to the many examples in the literature of the adverse effects on conservation management of disease introduction. For example, avian malaria was introduced via translocation of an infected individual into a threatened African black-footed penguin (*Spheniscus demersus*) population (Cranefield *et al*., 1994) and infection of *Cryptosporidium muris*, a species uncommon in marsupials, was found in captive bred greater bilbies (*Macrotis lagotis*) meant for reintroduction into the wild as part of a recovery program in Perth, Australia (Warren *et al*., 2003). Due to these risk factors, the use of anti-parasite drugs such as anti-coccidials and anthelmintics can be a common procedure in captive facilities (Fayer, 1980, Arias *et al*., 2013, Lott *et al*., 2014, Robertson *et al*., 2015). However, studies have shown that the practice of administering anti-parasitics to captive animals or animals meant for release may have a cost that outweighs the benefits. Including the added risk of developing anthelminthic resistence in the parasite populations (Lott *et al*., 2014, Robertson *et al*., 2015). Furthermore,

while anthelmintics may lower the infection intensity of the target parasite, the use of these drugs may increase the infection intensity of non-target organisms such as coccidians (Knowles *et al*., 2013, Pedersen & Antonovics, 2013).

Despite the role of parasites in potential disease, growing evidence suggests that maintaining host-parasite relationships is important to the success of conservation of threatened wildlife. Such practice also considers the parasite community part of overall biodiversity (Dobson *et al*., 2008) and as a driver of host biodiversity (Gómez & Nichols, 2013).

1.2 The importance of specialised parasites to biodiversity and conservation

The host-parasite relationship is an important driver of biodiversity for both the host and the parasite (Nieberding *et al*., 2008). In an evolutionary arms race, the parasite needs to invade its host, evade the immune system and reproduce within and between hosts. In response to the parasite, the host may change its behaviour or immune system to overcome and expel the parasite (Paterson & Piertney, 2011). Subsequently, some parasites may become highly specialised to a particular host species and thus become highly host specific or specialist parasites (Koh *et al*., 2004b). As specialist parasites may evolve to have low pathogenicity in the host and yet stimulate the host immune response, the presence of specialist parasites may lower the infection success of invasive, generalist parasites (Egas *et al*., 2004, Roth *et al*., 2012, Knowles *et al*., 2013, Desjardins *et al*., 2015). The presence of specialist parasites thus may have influence on the overall health and biodiversity of the host population.

Specialist parasites are bound by host specificity to host species or populations, particularly if those host populations are fragmented with limited gene flow (Thompson, 1999, Froeschke *et al*., 2013). Geography may further limit parasite distribution as certain conditions may be required for the parasite life cycle, e.g. water or a specific vector (Thompson, 1999). Due to these host population and geographical boundaries, host specific parasite communities can be

altered or negatively affected by conservation management strategies such as translocation (Moir *et al*., 2012). The restrictions on specialist parasite communities also imply that if their host becomes extinct, the entire parasite community will also go extinct, a process called coextinction (Dunn *et al*., 2009). Therefore, the loss of a threatened animal has greater consequences to the biodiversity and ecology of an environment than the loss of a single species (Koh *et al*., 2004a).

As an imbalance in the host-parasite relationship may impact the health of the host and the overall biodiversity of an ecosystem, more knowledge is required on how parasite communities of threatened animals are affected by conservation management. To fill this knowledge gap, studies need to examine the prevalence and diversity of parasites within populations of a threatened animal that is part of a conservation management program. Conservation strategies should then consider and manage the parasite communities of threatened animals based on the outcomes of these studies.

1.3 The brush-tailed rock-wallaby (*Petrogale penicillata*) and its conservation management program

The brush-tailed rock-wallaby (BTRW), *Petrogale penicillata* (Marsupialia: Macropodidae) is a 6-8 kg marsupial (Fig. 1.1) belonging to the genetically diverse genus *Petrogale* (Eldridge, 2008). Seventeen *Petrogale* species are currently recognised, which is the highest number of living species within any genus of the family Macropodidae – kangaroos and wallabies (Eldridge, 2008, Potter *et al*., 2015). Rock-wallabies live and forage in steep rocky habitats (Eldridge, 2008). Their adaptation to this niche is likely to have driven their high genetic diversity, which is unusual among other vertebrates (Eldridge & Close, 1993, Eldridge & Metcalfe, 2006).

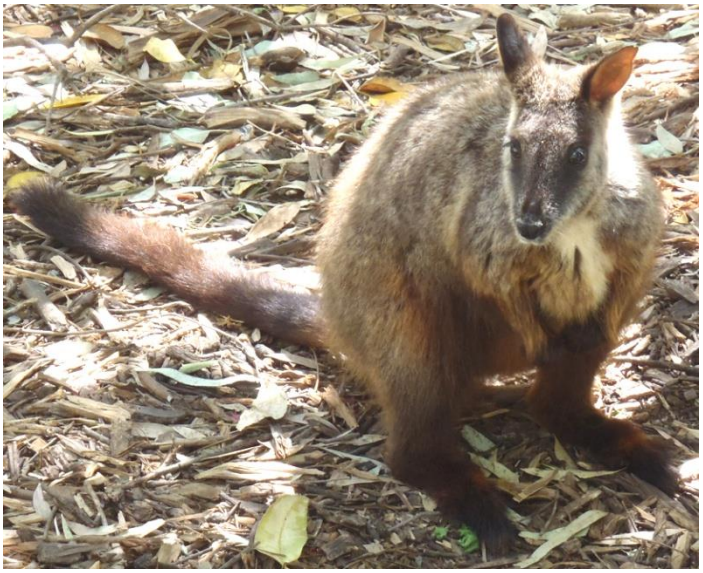

Figure 1.1: A brush-tailed rock-wallaby from Featherdale Wildlife Park, NSW (Photo by Elke Vermeulen)

The BTRW was once abundant throughout south-eastern Australia from south-east Queensland to western Victoria (Fig. 1.2, Eldridge & Close, 2005). However, since the European settlement of Australia, their numbers have been greatly diminished due to hunting, habitat destruction, competition with introduced herbivores and predation by introduced carnivores such as cats and foxes (Short & Milkovits, 1990, Dovey *et al*., 1997, Lunney *et al*., 1997). The total population of BTRW is estimated to be between 15,000 and 30,000 individuals (Taggart *et al*., 2008b), which are divided amongst three evolutionarily significant units (ESU) (Browning *et al*., 2001, Hazlitt *et al*., 2014).

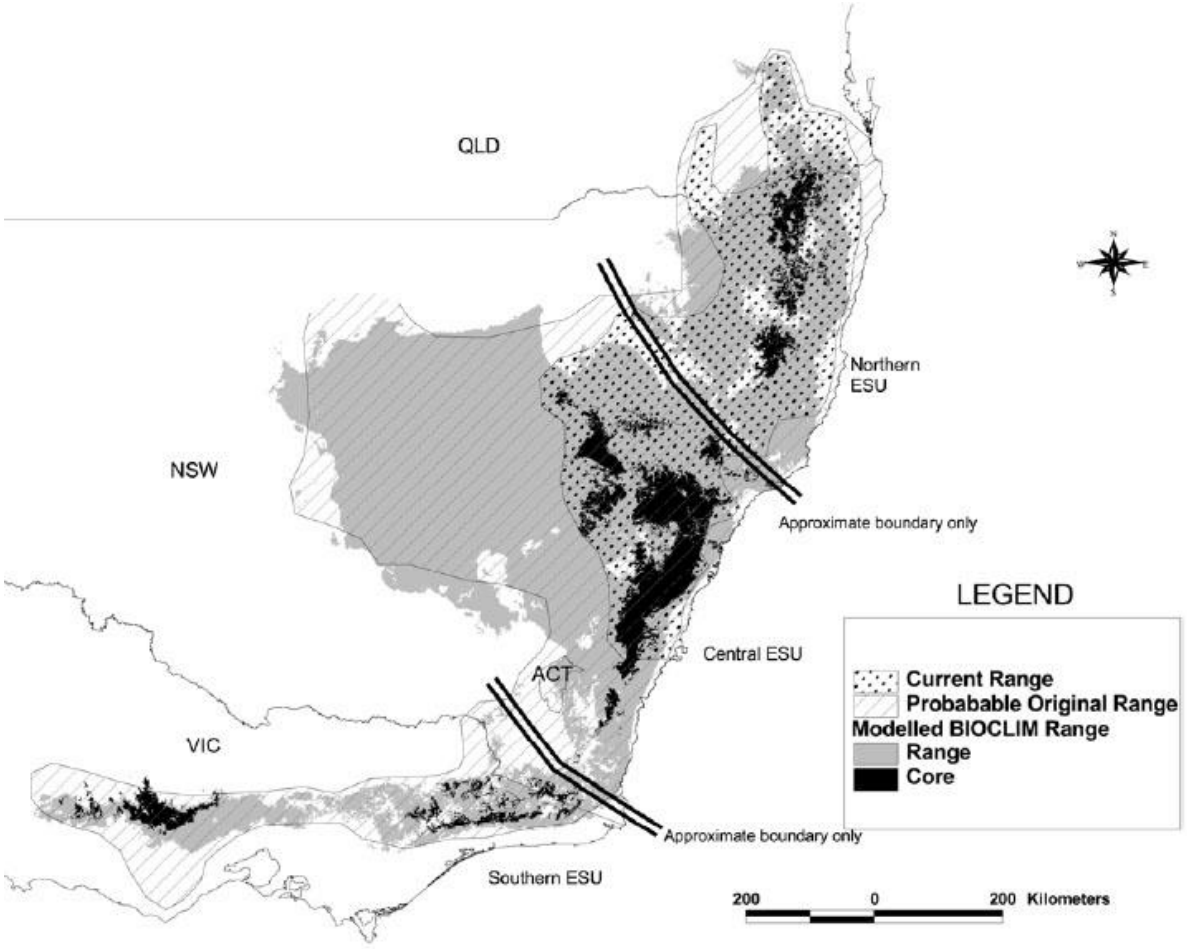

Figure 1.2: The probable historical range and current distribution (wild populations) of the BTRW, divided into the three ESUs across south-eastern Australia (DECC, 2008).

Brush-tailed rock-wallabies have been reduced to small, fragmented populations, particularly in New South Wales and Victoria (Eldridge & Close, 2005). Movement of animals between these populations is rare or impossible (Browning *et al*., 2001, Hazlitt *et al*., 2006), hence there is limited gene flow and decreasing genetic diversity within these small populations (Eldridge *et al*., 1999, 2004, Hazlitt *et al*., 2006). Reduced population size and low genetic diversity lead to inbreeding, which reduces fitness, and increases the risk for extinction (Frankham, 1995, Hedrick & Kalinowski, 2000). Low genetic diversity makes the host more vulnerable to disease and parasite invasion, particularly from invasive or generalist pathogens (Thompson *et al*., 2010a, King & Lively, 2012).

The BTRW is considered ‘Near Threatened’ on the International Union for Conservation of Nature (IUCN) Red List for Threatened Species (IUCN, 2015). In New South Wales, BTRW are listed as ‘endangered’ (NSW *Threatened Species Conservation Act* 1995) and in Victoria, ‘critically endangered’ with most natural populations now extinct (Menkhorst & Hynes, 2010, DSE, 2007). In 2008, a National Recovery Plan for the BTRW was compiled, listing the threats and the conservation actions to be undertaken (DECC, 2008). To increase the size and number of BTRW populations, a number of captive breeding and translocation programs has been conducted in New South Wales and Victoria (DECC, 2008, Taggart *et al*., 2008a, Menkhorst & Hynes, 2010, Schultz *et al*., 2011, Soderquist, 2011). In the current captive breeding and translocation program in New South Wales, which is the program examined in this thesis, no anti-parasite (anti-coccidials or anthelmintics) were used during captivity or before translocation. The potential impact of parasites on the success of the conservation management of BTRW is currently unknown but may be significant to conservation outcomes.

1.4 The diversity of specialist and generalist protozoan parasites in marsupials

Any potential disturbance in the host-parasite relationship due to conservation outcomes on parasite communities would potentially be stronger on host specific parasites than on those that can transfer between different host species (Moir *et al*., 2012). As parasites form a complex community within the host, a disturbance in the relationship between the host and specialist parasites with low pathogenicity may allow for greater infection success of invasive, generalist parasites (Sousa 1991, Holmes, 1996, Knowles *et al*., 2013, Desjardins *et al*., 2015). The protozoan parasite *Eimeria* is typically highly host specific (Duszynski & Wilber, 1997), while *Cryptosporidium* has species with varying host specificity (Ryan *et al*., 2014) and *Giardia duodenalis* is generally broad in its host range (Cacciò & Ryan, 2008, Feng & Xiao, 2011). All three parasites have the potential to be pathogenic, resulting in

coccidiosis, cryptosporidiosis and giardiasis respectively, and pathogenicity is potentially related to infection intensity (Daugschies & Najdrowski, 2005) and the immune status of the host (Barker *et al*., 1972, Thompson *et al*., 1993, Bettiol *et al*., 1997, Thompson *et al*., 2005, Xiao & Feng, 2008). Knowledge of the pathology of these parasites in wild or captive marsupials is lacking with few studies to date having examined symptomatic cases (Barker *et al*., 1972, Hum *et al*., 1991, Bettiol *et al*., 1997). All three parasites have an infective stage in their lifecycle found in the host's faeces, the oocyst, which contains the infective sporozoites (*Eimeria* and *Cryptosporidium*), and the cyst (*Giardia duodenalis*) (Marquardt *et al*., 2000). As this study aimed to be non-invasive due to the threatened status of the study host (*Petrogale penicillata*), the infective stages of the parasites' lifecycle within the faeces were used for parasite analysis and identification.

1.4.1 *Eimeria*: a specialist parasite

Eimeria (Apicomplexa: Eimeriidae) is a highly diverse coccidian parasite infecting a large range of vertebrates (Blake, 2015). *Eimeria* is highly host specific (oioxenous), with species either infecting one host species or only closely related species or genera (stenoxenous) (Barker *et al*., 1988c, Zhao *et al*., 2001, Kvičerová & Hypša, 2013). Within marsupials, *Eimeria* is highly host specific within marsupial genera (Table 1.1). The life cycle of *Eimeria* involves a transfer between hosts through the faecal-oral route. The stage of the life cycle within the host gut, the merozoite, is obligatory intracellular and the production of gametes through sexual reproduction also occurs within the epithelial cells of the host gut prior to release of oocysts in the faeces (Marquardt *et al*., 2000). Typically, *Eimeria* species have been identified by the morphology of the sporulated oocyst, including characteristics of the sporocysts and sporozoites (Fig. 1.3) (Duszynski & Wilber, 1997).

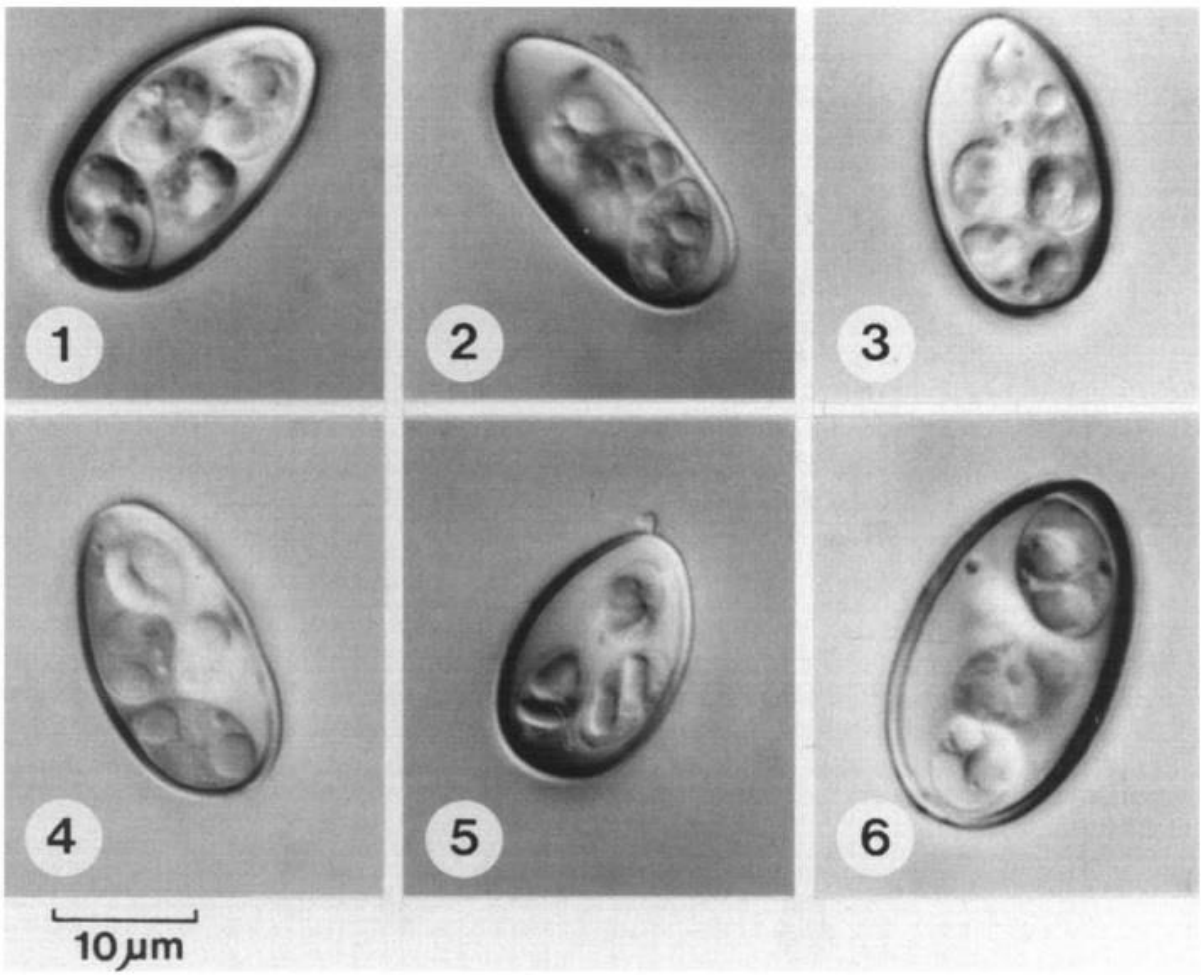

Figure 1.3: The sporulated oocyst of various *Eimeria* species in *Petrogale* hosts (Barker *et al*., 1988c). The scale is the same for all oocyst images. The oocysts are *Eimeria petrogale* in *P. assimilis* (1), *P. inornata* (2), *P. godmani* (3), *P. penicillata* (4) and *P. lateralis* (5), and *Eimeria boonderooensis* in *P. assimilis* (6). The *Petrogale* species names noted here reflect those as named in the study (Barker *et al*., 1988c), and prior to the taxonomic revision of the host genus in 1992 (Eldridge & Close, 1992).

Species identification of *Eimeria* using oocyst morphology can be complicated by mixed infections, where multiple species simultaneously infect an individual host (Barker *et al*., 1988c, Duszynski & Wilber, 1997, Jeanes *et al*., 2013). Oocyst characteristics can be polymorphic within a single species (Hill *et al*., 2012), and conversely, some species share highly similar morphological traits (Long & Joyner, 1984, Zhao *et al*., 2001). Due to the

difficulties posed by traditional methods of identification of *Eimeria* species, recent studies have aimed to supplement the current morphology-based knowledge with genetic data (Power *et al*., 2009b, Hill *et al*., 2012, Heitlinger *et al*., 2014, Reid *et al*., 2014, Blake, 2015). While 47 species of *Eimeria* have been described in marsupials (Table 1.1), only four of these have been defined using genotyping: *Eimeria macropodis*, *E. quokka*, *E. setonicis* and *E. trichosuri* (Power *et al*., 2009b, Hill *et al*., 2012, Austen *et al*., 2014). No genetic study has been conducted on *Eimeria* in BTRW. Three species of *Eimeria* (*E. inornata*, *E. petrogale* and *E. sharmani*) have been described from BTRW using morphological traits (Barker *et al*., 1988c).

Table 1.1: *Eimeria* species described in marsupial hosts, grouped by host family.

***Eimeria* species**	**Host Family**	**Host species**	**Reference**
Eimeria bicolor	Macropodidae	*Wallabia bicolour*	Barker *et al*., 1988b
Eimeria boonderooensis	Macropodidae	*Petrogale assimilis, Petrogale inornata, Petrogale mareeba, Petrogale sharmani*	Barker *et al*., 1988c
Eimeria dendrolagi	Macropodidae	*Dendrolagus lumholtzi*	Barker *et al*., 1988b
Eimeria desmaresti	Macropodidae	*Macropus rufogrieseus*	Barker *et al*., 1989
Eimeria flindersi	Macropodidae	*Macropus antilopinus, Macropus eugenii, Macropus rufogriseus*	Barker *et al*., 1989
Eimeria godmani	Macropodidae	*Petrogale assimilis, Petrogale godmani, Petrogale inornata, Petrogale lateralis*	Barker *et al*., 1988c, O'Callaghan *et al*., 1998, Duszynski, 2015
Eimeria gungahlinensis	Macropodidae	*Macropus fuliginosus, Macropus giganteus*	Barker *et al*., 1989
Eimeria hestermani	Macropodidae	*Macropus dorsalis, Macropus eugenii, Macropus fuliginosus, Macropus giganteus, Macropus rufogriseus*	Barker *et al*., 1989
Eimeria inornata	Macropodidae	*Petrogale inornata, Petrogale lateralis, Petrogale penicillata*	Barker *et al*., 1988c, O'Callaghan *et al*., 1998
Eimeria kogoni	Macropodidae	*Macropus giganteus*	Barker *et al*., 1989
Eimeria lagorchestis	Macropodidae	*Lagorchestes conspicillatus*	Barker *et al*., 1988b
Eimeria lumholtzi	Macropodidae	*Dendrolagus lumholtzi*	Barker *et al*., 1988b
Eimeria macropodis	Macropodidae	*Macropus dorsalis, Macropus eugenii, Macropus fuliginosus, Macropus giganteus, Macropus irma, Macropus parma, Macropus parryi, Macropus rufogriseus, Macropus rufus*	Barker *et al*., 1989
Eimeria marsupialium	Macropodidae	*Macropus fuliginosus, Macropus giganteus*	Barker *et al*., 1989
Eimeria mykytowyczi	Macropodidae	*Macropus agilis, Macropus antilopinus, Macropus parryi*	Barker *et al*., 1989
Eimeria obendorfi	Macropodidae	*Thylogale billardierii*	Barker *et al*., 1988b
Eimeria occidentalis	Macropodidae	*Petrogale inornata, Petrogale penicillata, Petrogale wilkinsi*	Barker *et al*., 1988c
Eimeria parryi	Macropodidae	*Macropus parryi*	Barker *et al*., 1989
Eimeria petrogale	Macropodidae	*Petrogale assimilis, Petrogale godmani, Petrogale herberti, Petrogale inornata, Petrogale lateralis, Petrogale mareeba, Petrogale penicillata, Petrogale persephone, Petrogale sharmani*	Barker *et al*., 1988c, Close *et al*., 1988, Begg *et al*., 1995, O'Callaghan *et al*., 1998
Eimeria prionotemni	Macropodidae	*Macropus agilis, Macropus dorsalis, Macropus eugenii, Macropus parryi, Macropus rufogriseus*	Barker *et al*., 1989
Eimeria quokka	Macropodidae	*Setonix brachyurus*	Barker *et al*., 1988b
Eimeria ringaroomaensis	Macropodidae	*Thylogale billardierii*	Barker *et al*., 1988b
Eimeria rufusi	Macropodidae	*Macropus rufus*	Barker *et al*., 1989

Table 1.1 Continued

***Eimeria* species**	**Host Family**	**Host species**	**Reference**
Eimeria setonocis	Macropodidae	*Setonix brachyurus*	Barker *et al*., 1988b
Eimeria sharmani	Macropodidae	*Petrogale assimilis, Petrogale godmani, Petrogale herberti, Petrogale inornata, Petrogale lateralis, Petrogale mareeba, Petrogale penicillata, Petrogale persephone, Petrogale rothschildi, Petrogale sharmani*	Barker *et al*., 1988c, Close *et al*., 1988, Begg *et al*., 1995, O'Callaghan *et al*., 1998
Eimeria thylogale	Macropodidae	*Thylogale billardierii*	Barker *et al*., 1988b
Eimeria toganmainensis	Macropodidae	*Macropus eugenii, Macropus fuliginosus, Macropus giganteus, Macropus rufogriseus, Macropus rufus*	Barker *et al*., 1989
Eimeria volckertzooni	Macropodidae	*Setonix brachyurus*	Barker *et al*., 1988b
Eimeria wallabiae	Macropodidae	*Wallabia bicolour*	Barker *et al*., 1988b
Eimeria wilcanniensis	Macropodidae	*Macropus fuliginosus, Macropus giganteus, Macropus robustus, Macropus rufus*	Barker *et al*., 1989
Eimeria xanthopus	Macropodidae	*Petrogale xanthopus*	Barker *et al*., 1988c
Eimeria yathongensis	Macropodidae	*Macropus fuliginosus, Macropus giganteus*	Barker *et al*., 1989
Eimeria aepyprymni	Potoroidae	*Aepyprymnus rufescens*	Barker *et al*., 1988a
Eimeria gaimardi	Potoroidae	*Bettongia gaimardi*	Barker *et al*., 1988a
Eimeria hypsiprymodontis	Potoroidae	*Hypsiprymodon moschatus*	Barker *et al*., 1988a
Eimeria kairiensis	Potoroidae	*Hypsiprymodon moschatus*	Barker *et al*., 1988a
Eimeria mundayi	Potoroidae	*Potorous tridactylus*	Barker *et al*., 1988a
Eimeria potoroi	Potoroidae	*Potorous tridactylus*	Barker *et al*., 1988a
Eimeria speari	Hypsiprymnodontidae	*Hypsiprymnodon moschatus*	Barker *et al*., 1988a
Eimeria spratti	Hypsiprymnodontidae	*Hypsiprymnodon moschatus*	Barker *et al*., 1988a
Eimeria tinarooensis	Hypsiprymnodontidae	*Hypsiprymnodon moschatus*	Barker *et al*., 1988a
Eimeria arundeli	Vombatidae	*Vombatus ursinus*	Barker *et al*., 1979
Eimeria wombati	Vombatidae	*Lasiorhinus latifrons*	Barker *et al*., 1979
Eimeria ursini	Vombatidae	*Lasiorhinus latifrons*	Barker *et al*., 1979
Eimeria trichosuri	Phalangeridae	*Trichosurus vulpecula*	O'Callaghan & O'Donoghue, 2001
Eimeria quenda	Peramelidae	*Isoodon obesulus*	Bennett & Hobbs, 2011
Eimeria kanyana	Peramelidae	*Perameles bougainville*	Bennett *et al*., 2006

1.4.2 *Cryptosporidium*: a parasite with varying host specificity between species

Cryptosporidium (Apicomplexa: Cryptosporidiidae) is a coccidian parasite that can infect over 150 vertebrate hosts (Fayer, 2010). The life cycle of *Cryptosporidium* is similar to *Eimeria*, including the transference of infective oocysts between hosts through the faecal-oral route (Marquardt *et al*., 2000). Unlike *Eimeria* oocysts, the *Cryptosporidium* oocyst contains no sporocysts and four sporozoites contained within the oocyst wall (Bouzid *et al*. 2013). Identification of *Cryptosporidium* species based on morphology of the oocyst is problematic since many species share highly similar morphological characteristics (Xiao *et al*., 2004, Chalmers & Davies, 2010, Fayer, 2010) (Fig. 1.4). *Cryptosporidium* species vary greatly in pathogenicity and host specificity with some species having a broad host range (euryxenous) and thus identification using molecular techniques has become essential (Xiao *et al*., 2004, Fayer, 2010).

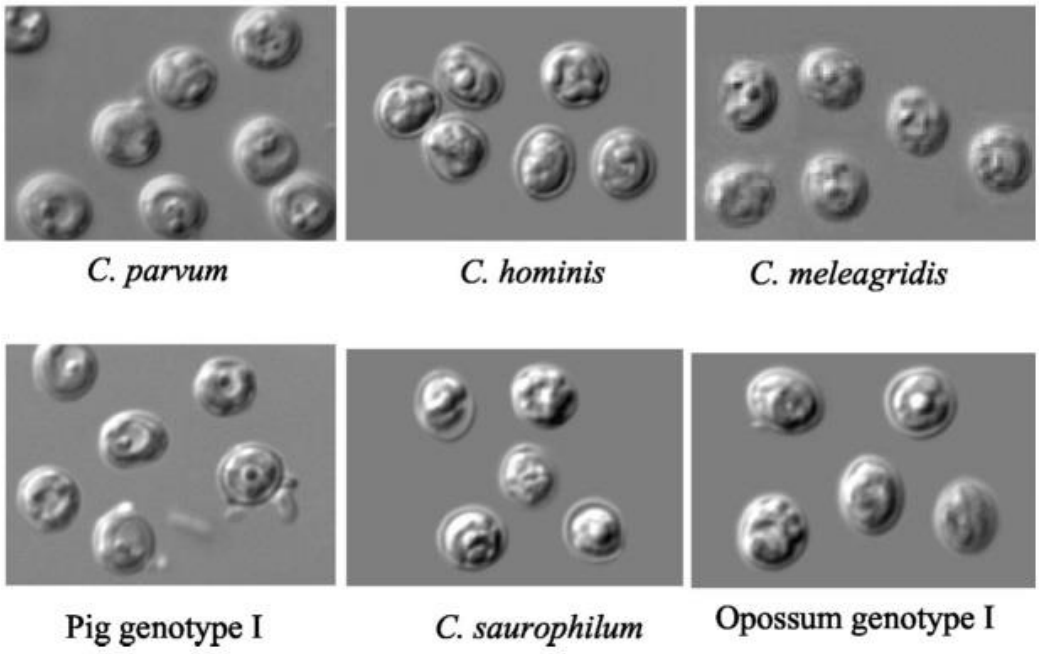

Figure 1.4: *Cryptosporidium* oocysts from various hosts, showing high similarity in oocyst morphology between species (Xiao *et al*., 2004).

Currently, 27 *Cryptosporidium* species have been described through genotyping, with more cryptic species known (Ryan *et al*., 2014, 2015). *Cryptosporidium hominis* and *C. parvum* are the most common species identified in human clinical cases, followed by *C. meleagridis* (Xiao & Feng, 2008). While *C. hominis* is host specific and occurring in humans, *C. parvum* and *C. meleagridis* have a broader host range and typically infect domestic animals such as cattle and poultry (Ryan *et al*., 2014). *Cryptosporidium* has been described in 16 Australian marsupial species across 7 families (Table 1.2); with *C. fayeri* and *C. macropodum* the two most commonly identified species (Power & Ryan, 2008, Ryan *et al*., 2008). Of twelve *Cryptosporidium* species known to be zoonotic (Xiao *et al*., 2004, Chalmers & Davies, 2010, Robinson *et al*., 2010, Waldron *et al*., 2010, Ryan & Power, 2012), only *C. fayeri* (Waldron *et al*., 2010) and *C. muris* (Warren *et al*., 2003) have been identified in marsupials. *Cryptosporidium* samples from bandicoots, possums and kangaroos have previously been identified as *C. parvum* or *C. hominis*-like but these were sequenced only at the 18S rRNA locus and with unsuccessful amplification of several other loci, thus lacking the more robust identification through multi-locus genotyping (Hill *et al*., 2008, Ng *et al*., 2011, Dowle *et al*., 2013). The failure to amplify *Cryptosporidium* specific loci suggests that the presence of *C. parvum and C. hominis* may be mechanical passage rather than a true infection in these marsupial cases (Dowle *et al*., 2013). The only *Cryptosporidium* infection identified within the genus *Petrogale* to date has been the identification of *C. fayeri* in the yellow-footed rock-wallaby (*P. xanthopus*) (Power *et al*., 2003, 2009a).

Table 1.2: Identifications of *Cryptosporidium* species in Australian marsupials grouped by host family. 'Unidentified' denotes cases where identification was made on morphological identification prior to the use of molecular analysis to identify species.

***Cryptosporidium* species**	**Host Family**	**Host Species**	**Reference**
Unidentified	Dasyuridae	*Antechinus stuartii*	Barker *et al*., 1978
C. fayeri and *C. macropodum*	Macropodidae	*Macropus giganteus*	Power *et al*., 2003, 2004
C. fayeri	Macropodidae	*Macropus rufus*	O'Donoghue, 1995
Unidentified	Macropodidae	*Thylogale billardierii*	O'Donoghue, 1995
Unidentified	Macropodidae	*Thylogale thetis*	Power *et al*., 2003
C. fayeri	Macropodidae	*Petrogale xanthopus*	Power *et al*., 2003
Unidentified	Macropodidae	*Macropus rufogriseus*	Power, 2002
C. fayeri and *C. macropodum*	Macropodidae	*Macropus fuliginosus*	McCarthy *et al*., 2008
C. macropodum	Macropodidae	*Wallabia bicolor*	reviewed in Power, 2010
Unidentified	Peramelidae	*Isoodon obesulus*	O'Donoghue, 1995
C. fayeri	Peramelidae	*Peremeles bougainville*	reviewed in Power, 2010
Unconfirmed *C. parvum* like	Peramelidae	*Perameles nasuta*	Dowle *et al*., 2013
Brushtail possum genotypes I and II	Phalangeridae	*Trichosurus vulpecula*	Power *et al*., 2003, Hill *et al*., 2008
C. fayeri	Phascolarctidae	*Phascolarctos cinereus*	Morgan *et al*., 1997
C. muris	Thylacomyidae	*Macrotis lagotis*	Warren *et al*., 2003
Unidentified	Vombatidae	*Vombatus ursinus*	Power, 2002

1.4.3 *Giardia duodenalis*: a species complex with generalist assemblages

Giardia (Diplozoa: Giardiidae) is a flagellated protozoan genus comprised of six species with species distinction based on the morphology of the trophozoite (Fig. 1.5), which is the life cycle stage within the host gut (Adam, 2001, Plutzer *et al*., 2010). The trophozoite is motile and uses an adhesive disk to adhere to the host's luminal surface (Adam, 2001). While *Giardia* reproduces asexually through binary fission, it produces hardy cysts (Fig. 1.5) that are excreted by the host in faeces (Marquardt *et al*., 2000, Adam, 2001). Five *Giardia* species are highly host specific while the sixth species, *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*) consists of multiple genetic strains infecting a large variety of mainly mammalian hosts and thus has a broad host range (euryxenous) (Plutzer *et al*., 2010). *Giardia*

duodenalis is considered a species complex and is divided into at least eight assemblages (A-H) initially based on their genetic characteristics at various loci (Feng & Xiao, 2011).

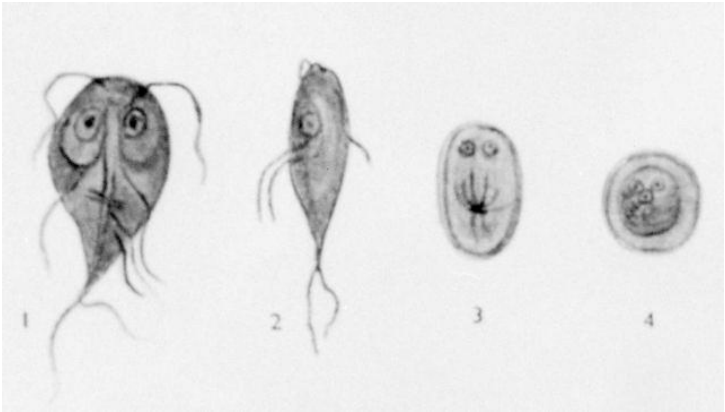

Figure 1.5: *Giardia duodenalis* trophozoites (1-2) and cysts (3-4). Image made by the Tropical Medicine Central Resource (copyright: Palmer and Reeder by Springer).

Both assemblages A and B have been regularly identified in many non-human animals, indicating these are anthropozoonotic assemblages (Cacciò & Ryan, 2008). More thorough genetic analysis has further divided the assemblages into sub-assemblages, e.g. AI to AIV, which vary in their host range and pathogenicity (Read *et al*., 2002, Feng & Xiao, 2011). *Giardia duodenalis* has been identified in 27 species within eight families of marsupials (Table 1.4). The most common assemblages of *G. duodenalis* recorded in marsupials are A and B (McCarthy *et al*., 2008, Thompson *et al*., 2008, 2010b, Ng *et al*., 2011). Identification of other assemblages, namely C, D and E, are rare but have been recorded in marsupials (Thompson *et al*., 2010b, Ng *et al*., 2011). *Giardia duodenalis* has not yet been identified in BTRW, but has been recorded in the yellow-footed rock-wallaby and was assigned to assemblages A and B (Thompson *et al*., 2008).

Table 1.3: Identifications of *Giardia duodenalis* in Australian marsupial species, grouped by host family. 'Unidentified' denotes cases where the identification was made based on morphological identification prior to the use of molecular analysis to identify species.

***G. duodenalis* assemblages**	**Host Family**	**Host species**	**Reference**
B	Dasyuridae	*Dasyurus maculatus*	Thompson *et al*., 2008
A	Dasyuridae	*Planigale maculata*	Thompson *et al*., 2010b
Unidentified	Dasyuridae	*Sarcophilus harrisii*	Millstein & Goldsmith, 1997
Unidentified	Dasyuridae	Unidentified	Bettiol *et al*., 1997
A and B	Macropodidae	*Macropus eugenii*	Thompson *et al*., 2008
A and B	Macropodidae	*Macropus fuliginosis*	Thompson *et al*., 2008
C	Macropodidae	*Macropus giganteus*	Ng *et al*., 2011
A and B	Macropodidae	*Macropus parma*	Thompson *et al*., 2008
Unidentified	Macropodidae	*Macropus rufogiseus*	Bettiol *et al*., 1997
A and B	Macropodidae	*Macropus rufus*	Thompson *et al*., 2008
A, B and D	Macropodidae	*Macropus* sp. (unidentified)	Ng *et al*., 2011
A and B	Macropodidae	*Petrogale xanthopus*	Thompson *et al*., 2008
A and B	Macropodidae	*Setonix brachyurus*	Thompson *et al*., 2008
Unidentified	Macropodidae	*Thylogale billardieri*	Bettiol *et al*., 1997
Human-infective assemblage	Macropodidae	*Thylogale thetis*	Buckley *et al*., 1997
A and B	Macropodidae	*Wallabia bicolor*	Thompson *et al*., 2008
Human-infective assemblage	Peramelidae	*Perameles gunnii*	Bettiol *et al*., 1997
Human-infective assemblage	Peramelidae	*Isoodon macrourys*	Buckley *et al*., 1997
Quenda genotype and E	Peramelidae	*Isoodon obesulus*	Adams *et al*., 2004, Thompson *et al*., 2010b
A	Phalangeridae	*Trichosurus vulpecula*	Marino *et al*., 1992, Thompson *et al*., 2008
A	Phalangeridae	*Trichosurus cunninghami*	Thompson *et al*., 2008
A	Phascolarctidae	*Phascolarctos cinereus*	Thompson *et al*., 2008
A	Potoroidae	*Potorous tridactylus*	Bettiol *et al*., 1997, Thompson *et al*., 2008
A	Potoroidae	*Aepyprymnus rufescens*	Thompson *et al*., 2008
Unidentified	Pseudocheiridae	*Pseudocheirus peregrinus*	Bettiol *et al*., 1997
Unidentified	Vombatidae	*Vombatus ursinus*	Bettiol *et al*., 1997
A	Vombatidae	*Lasiorhinus latifrons*	Thompson *et al*., 2008

1.5 Detection and identification of enteric protozoan parasites

As all three parasites (*Eimeria*, *Cryptosporidium* and *Giardia duodenalis*) are excreted as oocysts / cysts in the faeces of the host (Marquardt *et al*., 2000), detection can occur non-intrusively by examining faecal matter for the target parasite. Historically, these parasites were detected using microscopy and then identified according to morphological traits (Barker *et al*., 1988c, Adam, 2001, Power *et al*., 2003). Identification through morphology alone is problematic for *Eimeria* and *Cryptosporidium* as many species share morphological attributes (Long & Joyner, 1984, Chalmers & Katzer, 2013). The assemblages of *Giardia duodenalis* are morphologically indistinguishable as per definition, they are grouped as a species complex under the species name *G. duodenalis* based on their morphology (Adam, 2001). Therefore, microscopy cannot distinguish between different assemblages of *G. duodenalis* and other techniques are required for this distinction.

Molecular techniques have expanded our knowledge of the diversity of parasites, particularly in wildlife. Polymerase chain reaction (PCR) has been found to be more sensitive to detect the presence of *Eimeria* (Austen *et al*., 2014), *Cryptosporidium* (Morgan & Thompson, 1998, Dowle *et al*., 2013) and *Giardia* (Ryan *et al*., 2005, Thompson *et al*., 2008, Asher *et al*., 2012) in faecal samples than conventional microscopy. As the small ribosomal subunit (18S rRNA) is highly conserved within taxonomic groups yet has hypervariable regions that can discriminate between genera, this locus is commonly used as a PCR target for eukaryotes (Gagnon *et al*., 1996, Morrison *et al*., 2004). Discrimination between species, however, is not robust when using only the 18S rRNA locus as this gene is highly conserved (Read *et al*., 2004, Power *et al*., 2009a, El-Sherry *et al*., 2013). Accurate identification of species or assemblages requires multi-locus genotyping (Cacciò & Ryan, 2008, Power *et al*., 2009a, Ogedengbe *et al*., 2011, Asher *et al*., 2012). Additional target loci for *Eimeria* are typically cytochrome c oxidase subunit I (*COI*) (Ogedengbe *et al*., 2011) and the internal transcribed

spacer (*ITS-1*) region of the rRNA locus (Hnida and Duszynski, 1999). Specific loci for *Cryptosporidium* include actin (Sulaiman *et al*., 2000) and glycoprotein 60kDA (*gp60*) (Strong *et al*., 2000, Winter *et al*., 2000). Specific identifications of *G. duodenalis* assemblages and sub-assemblages have previously been made at the glutamate dehydrogenase (*gdh*) (Read *et al*., 2004) and the β-giardin (Cacciò *et al*., 2002) loci.

Conventional PCR and Sanger sequencing will, however, underestimate the presence of mixed infections due to the detection of only the strongest signal in a sample (Sanger *et al*., 1977, Reed *et al*., 2002, Cacciò & Ryan, 2008, Geurden *et al*., 2008). Mixed infections of *Cryptosporidium* are rare (Cama *et al*., 2006) but are more common in *G. duodenalis*, particularly the closely related A and B assemblages (Cacciò & Ryan, 2008). Mixed infections are also common in the genetically diverse *Eimeria* (Barker *et al*., 1988c, Jeanes *et al*., 2013, Blake, 2015). To study the community structure of bacteria, next generation sequencing (NGS) is commonly utilised as a sensitive and accurate tool (Faust & Raes, 2012, Quail *et al*., 2012). These sequencing techniques have recently been utilised to investigate complex community structures of eukaryotes (Lecroq *et al*., 2011, Bálint *et al*., 2014, Lott *et al*., 2015), but not yet for analysing the community structures of protozoan parasites.

1.6 Research objectives

Conservation management strategies such as captive breeding and translocation may have an impact on the host-parasite relationship by lowering the genetic diversity of specialist parasites (Moir *et al*., 2012) or by introducing novel parasites through atypical contact with humans or other animals (Cunningham, 1996). The introduction of anthroponotic species or assemblages of parasites may be detrimental to the health of the host population (Cranefield *et al*., 1994). The maintenance of the community structure of specialist parasites contributes to the biodiversity of the ecosystem (Dobson *et al*., 2008) and the host-parasite relationship

increases the biodiversity of both hosts and parasites (Paterson & Piertney, 2011). To implement management strategies that consider the parasite communities of threatened animals, more knowledge is required of the impact of current management strategies on parasites in a host that is part of a conservation program.

The primary aim of this study was to investigate whether conservation management of BTRW had an impact on the prevalence and genetic diversity of its protozoan parasites. If the host-parasite balance was disturbed by conservation management practices, a difference in prevalence and genetic diversity of parasites may be observed in BTRW that had direct contact with humans compared to their wild conspecifics. To investigate the impact of conservation management on the host-parasite relationship of BTRW, this thesis had five main objectives:

1. To summarise the current knowledge of parasites in rock-wallabies (genus *Petrogale*) and determine the level of host specificity of the parasite communities in this highly diverse genus;
2. to compare the prevalence of *Cryptosporidium, Eimeria* and *G. duodenalis* between managed and wild BTRW populations;
3. to determine if conservation management of BTRW has introduced anthroponotic parasite species/assemblages though genetic analysis of *Cryptosporidium* and *Giardia*;
4. to develop analyses for examining the community structure of protozoan parasites using next generation sequencing (NGS), namely Illumina MiSeq; and
5. to apply the developed NGS methodology to investigate the genetic structure of *Eimeria* within and between managed and wild populations of BTRW

1.7 Positioning of thesis chapters

Chapter 1: General Introduction

This chapter explains the background and sets the justification for the thesis, namely why studying the effect of conservation management on the biodiversity of parasites is an important topic. It discusses the background of the host, the brush-tailed rock-wallaby (BTRW, *Petrogale penicillata*), its conservation status and current conservation management program in New South Wales. The chapter then introduces the three protozoan parasites examined in this thesis: *Eimeria, Cryptosporidium* and *Giardia duodenalis*, and their background in Australian marsupials in general and rock-wallabies in particular.

Chapter 2: Biodiversity of parasite assemblages in the genus *Petrogale* and its relation to the phylogeny and biogeography of their hosts.

Elke T. Vermeulen, David Nipperess, Ian Beveridge, Mark D. B. Eldridge, Michelle L. Power

Australian Journal of Zoology (2016) 64 (1), 61-80

Australia has high biodiversity and rock-wallabies (genus *Petrogale*) are one of the most genetically diverse genera of vertebrates. Much effort has been put into documenting the complex parasite communities of Australian wildlife thus far. However, the extent of the biodiversity and host specificity of parasite communities in marsupial hosts is mostly still unknown. Documenting the biodiversity of parasites is of particular importance as many marsupial species, including rock-wallabies, are currently threatened making their host specific parasites equally threatened. A database was created for all known parasite species recorded to date in *Petrogale* and used to analyse the host specificity and diversity of the *Petrogale* parasite communities.

Addresses objective 1

Chapter 3: Diversity of *Cryptosporidium* in brush-tailed rock-wallabies managed within a species recovery program (*Petrogale penicillata*)

Elke T. Vermeulen, Deborah L. Ashworth, Mark D. B. Eldridge, Michelle L. Power

International Journal of Parasitology: Parasites and Wildlife (2015) 4 (2), 190-196

Cryptosporidium is a protozoan parasite comprised of species with variable host specificity. While it is a significant pathogen in humans and domestic animals the pathogenicity in Australian marsupials is unknown. This chapter examines whether atypical contact with humans through conservation management facilitates transmission of human-associated species of *Cryptosporidium* and whether host specific *Cryptosporidium* species are present in captive bred and supplemented populations through investigation of the prevalence and genetic diversity of *Cryptosporidium* species in BTRW populations.

Addresses objective 2 and 3

Chapter 4: Investigation into potential transmission sources of *Giardia duodenalis* in a threatened marsupial (*Petrogale penicillata*).

Elke T. Vermeulen, Deborah L. Ashworth, Mark D. B. Eldridge, Michelle L. Power

Infection, Genetics and Evolution (2015) 33, 277-280

Giardia duodenalis is a species complex, divided into genetic assemblages (A-H) and sub-assemblages (e.g. AI-AIV) and these assemblages vary in their pathogenicity and host specificity. Previous studies have identified the human infective assemblages A and B in various wild animals, including Australian marsupials. This chapter describes an investigation of the prevalence of *G. duodenalis* in differently managed and wild populations

of BTRW and identifies of the assemblages and sub-assemblages of *G. duodenalis* in BTRW populations to determine if conservation management introduced *G. duodenalis* assemblages in captive bred BTRW.

Addresses objective 2 and 3

Chapter 5: Evaluation of next generation sequencing for the analysis of *Eimeria* communities in wildlife

Elke T. Vermeulen, Matthew J. Lott, Mark D. B. Eldridge, Michelle L. Power

Journal of Microbiological Methods (2016) 124, 1-9

To date few studies have examined the genetic diversity of *Eimeria* communities in Australian wildlife. While next-generation sequencing (NGS) has been previously utilised to study the genome of commercially important *Eimeria* species, no study has yet applied NGS to examine *Eimeria* communities. To undertake a genetic analysis of *Eimeria* communities in BTRW required a PCR protocol for the amplification of a region of the 18S rRNA locus suitable for NGS. The development of a methodology to analyse NGS data for *Eimeria* communities was also required. This chapter describes the development of methodologies to enable assessment of *Eimeria* community structure using NGS.

Addresses objective 4

Chapter 6: Parasites on the hop: captive breeding maintains biodiversity of *Eimeria* communities in an endangered marsupial

Elke T. Vermeulen, Matthew J. Lott, Mark D. B. Eldridge, Michelle L. Power

Biological Conservation (2016) 200, 17-25

After developing the methodology in chapter 5, it was applied to compare *Eimeria* communities from BTRW populations in different management categories (captive bred and supplemented) and wild populations to determine whether conservation management has an impact on specialist parasite communities.

Addresses objective 2 and 5

Chapter 7: General Discussion

This chapter summarises and discusses the key findings of this study, namely how the parasite communities of the BTRW were affected by the conservation management program. There is further discussion on the implications of the outcomes on the BTRW program in particular and wider implications for conservation management of wildlife. Furthermore, the methodologies employed in this thesis could be used to conduct similar, larger scale studies of parasite communities in endangered wildlife.

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Eimeria spp. from bats and rodents based on nuclear 18S and plastid 23S rDNA sequences. *Journal of Parasitology*, 87**,** 1120-1123.

2 Biodiversity of parasite assemblages in the genus *Petrogale* and its relation to the phylogeny and biogeography of their hosts

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Keywords: Biodiversity, parasitology, conservation genetics, phylogeny, population dynamics

Published in: *Australian Journal of Zoology* (2016) 64 (1), 61-80.

DOI: 10.1071/ZO16023

2.1 Abstract

Parasites form an integral part of overall biodiversity although they are often overlooked in conservation management, where emphasis is primarily directed towards the host. Parasites are often highly specialized to particular hosts, and thus may be just as threatened as the host they inhabit. Australia has highly endemic and diverse wildlife, with many species at risk from multiple anthropogenic factors. For many Australian wildlife species, little is known about their associated parasite communities. To begin to address this knowledge gap, we documented the parasite fauna described in the highly diverse marsupial genus *Petrogale*, which contains seven species of conservation concern. The literature evaluation showed parasites of *Petrogale* to be highly diverse with 17 species of protozoa, 8 species of cestodes, 102 species of nematodes and 30 species of ectoparasites described in 16 of 17 *Petrogale* host species. A comparison of the composition and similarity of the parasite communities amongst *Petrogale* host species indicated a highly significant correlation between the parasite community similarity, and the phylogeny ($p = 0.008$) and biogeography ($p = 0.0001$) of their *Petrogale* hosts, suggesting high host specificity within their associated parasites. Five *Petrogale* species have established species recovery programs and their parasite communities should also be considered threatened, and management of parasite diversity required as part of these conservation programs.

2.2 Introduction

Australia's unique biodiversity is declining as a result of global change, with habitat loss, the introduction of invasive species, human population expansion and climate change representing significant drivers of decline (NRMMC, 2010, Nipperess, 2015). The field of conservation biology aims to maintain biodiversity, including the evolutionary drivers of biodiversity (Meffe *et al*., 2006). Parasites have been recognised as important drivers of species diversification due to coevolution between host and parasite (Nieberding *et al*., 2008, Paterson & Piertney, 2011). However, wildlife conservation often only focuses on vertebrate organisms (Fowler & Miller, 2008) despite the complex community of parasites which may be integral to host evolution and also at risk of loss (Woodford, 2000, Rigaud *et al*., 2010). With emphasis on conservation of the wildlife host, parasites are either overlooked or only considered relative to disease management (Horan & Melstrom, 2011, Gómez & Nichols, 2013). The process of conservation management has also been shown to damage the dependent parasite community of the target organism (Moir *et al*., 2012) despite a growing body of evidence that parasites are important to maintaining biodiversity (reviewed in Gómez & Nichols, 2013). The management of parasites therefore needs to be considered within conservation strategies.

The host-parasite relationship is recognised as one of the major drivers of speciation through evolutionary adaptation (Nieberding *et al*., 2008). Parasites need to invade the host, evade its immune system, establish a population and then reproduce to continue their life-cycle, which in turn impacts upon host biology and behaviour, thus driving an antagonistic coevolution (Paterson & Piertney, 2011, Labaude *et al*., 2015). To have evolutionary advantage over the host and over other competitors, some parasites have become highly specialised (oioxenous) to their host population or to closely related sympatric hosts (stenoxenous) (Toft & Karter, 1990). This evolutionary adaptation can restrain the specialised parasite to a certain locality,

particularly if the host population has become fragmented or isolated (Kaltz & Shykoff, 1998, Lively & Dybdahl, 2000). The constraint of geographical location and host availability implies that if the host population were to become extinct, its specific parasite and commensal community would also die out (Dunn *et al*., 2009).

Coextinction describes the process of loss of affiliated species with the loss of one species, which can be its particular food source or host (Stork & Lyal, 1993). Particularly, host-specific parasites are in danger of coextinction if their host becomes extinct (Koh *et al*., 2004a). Moreover, highly specialized parasites have a greater potential to be endangered than their host, especially those parasites with a complex life cycle (Koh *et al*., 2004b, Pizzi, 2009). The close relationship between parasites and their hosts identifies the need to document parasite associations and the importance of this information to preserving biodiversity. While considerable efforts have been made to document the diversity of parasites of Australian wildlife (e.g. Spratt *et al*., 1991, Thompson *et al*., 2010a, Beveridge & Spratt, 2015), knowledge of the distribution and diversity of parasites in Australia remains limited, presenting risks for coextinction with threatened species (reviewed in Poulin, 2014).

2.3 The hosts: rock-wallabies

Rock-wallabies (genus *Petrogale*, Marsupialia: Macropodidae) are one of the largest groups of extant macropodoids (kangaroos, wallabies and potoroids) with 17 species currently recognised (Eldridge, 2008, Potter *et al*., 2015) (Table 2.1). Rock-wallabies are adapted to living in a variety of rocky habitats ranging from tropical rainforests to deserts across mainland Australia (Eldridge, 2008). Life in isolated rocky areas has resulted in morphological and behavioural adaptations, which are thought to have driven high genetic diversity, which is unusual among other vertebrates (Eldridge & Close, 1993, Eldridge & Metcalfe, 2006). Mitochondrial and nuclear DNA sequence analysis has defined four major

clades within *Petrogale*: the *lateralis-penicillata* group (11 species), the *brachyotis* group (4 species) and then two single-species lineages comprising *P. xanthopus* and *P. persephone* (Potter *et al*., 2012) (Table 2.1, Suppl. Fig. 2.1). Most *Petrogale* species within a clade are parapatric, except for the *brachyotis* group species where several species occur in sympatry. Conversely, most species that are less closely related phylogenetically are allopatric, suggesting a link between phylogeny and biogeography (Eldridge & Close, 1993, Potter *et al*., 2012) (Suppl. Fig. 2.2).

Table 2.1: The seventeen recognized rock-wallaby (*Petrogale*) species with their lineage affiliations (Eldridge 2008, Potter *et al*. 2015) and current conservation status.

Chromosomal group	**Common name**	**Scientific name**	**Status (IUCN 2015)****	**Status: EPBC Act*****
xanthopus	Yellow-footed rock-wallaby	*Petrogale xanthopus*	Near Threatened	Vulnerable
persephone	Proserpine rock-wallaby	*Petrogale persephone*	Endangered (B1ab (iii, v))	Endangered
brachyotis	Short-eared rock-wallaby	*Petrogale brachyotis*	Least Concern	Not listed
	Short-eared rock-wallaby	*Petrogale wilkinsi**	Not assessed	Not assessed
	Monjon	*Petrogale burbidgei*	Near Threatened	Not listed
	Nabarlek	*Petrogale concinna*	Data Deficient	Endangered
lateralis–penicillata	Black-footed rock-wallaby	*Petrogale lateralis*	Near Threatened	Vulnerable
	Purple-necked rock-wallaby	*Petrogale purpureicollis*	Least Concern	Not listed
	Rothschild's rock-wallaby	*Petrogale rothschildi*	Least Concern	Not listed
	Allied rock-wallaby	*Petrogale assimilis*	Least Concern	Not listed
	Cape York rock-wallaby	*Petrogale coenensis*	Near Threatened	Endangered
	Godman's rock-wallaby	*Petrogale godmani*	Least Concern	Not listed
	Herbert's rock-wallaby	*Petrogale herberti*	Least Concern	Not listed
	Unadorned rock-wallaby	*Petrogale inornata*	Least Concern	Not listed
	Mareeba rock-wallaby	*Petrogale mareeba*	Least Concern	Not listed
	Brush-tailed rock-wallaby	*Petrogale penicillata*	Near Threatened	Vulnerable
	Sharman's rock-wallaby	*Petrogale sharmani*	Near Threatened	Vulnerable

**Petrogale wilkinsi* was previously included within *P. brachyotis* (Potter *et al*. 2015)

** International Union for Conservation of Nature (IUCN) Red List 2015

*** Environment Protection and Biodiversity Conservation Act 1999

Of the 17 recognized *Petrogale* species, one is currently listed as Endangered and six as Near Threatened (IUCN, 2015) (Table 2.1). Those species of conservation concern have been reduced to fragmented populations with limited gene flow (Hazlitt *et al*., 2006, Pearson, 2012). Intensive conservation management including captive breeding and translocation of both captive-bred and wild-sourced individuals has been undertaken for five species: *P. lateralis*, *P. penicillata*, *P. persephone*, *P. rothschildi* and *P. xanthopus* (DECC, 2008, Pearson, 2012). Efforts to repopulate native habitats for *P. lateralis* and *P. rothschildi* have included 19 translocation events since 1960 (Pearson, 2012). Captive breeding and re-introduction or supplementation into remnant populations have been conducted for *P. penicillata* in New South Wales (Soderquist, 2011) and Victoria (Schultz *et al*., 2006, Taggart *et al*., 2008, 2009), for *P. persephone* in Queensland (Johnson *et al*., 2003) and for *P. xanthopus* in both South Australia (Barlow, 1999) and Queensland (Lapidge & Munn, 2011).

Rock-wallabies belong to the marsupial family Macropodidae which may harbour a more diverse parasite assemblage than any other major group of mammals (Beveridge & Chilton, 2001, Coulson & Eldridge, 2010). Therefore, we aim to provide a database of parasites identified to date in rock-wallabies and to define the extent of parasite diversity and specificity in this highly diverse host group containing many species of conservation concern.

2.4 Parasites of rock-wallabies

Identification of parasites is a complex process comprising a combination of methods. Morphological identifications of adult stages of parasites are usually from post-mortem examinations or blood smears, while the infective stages can often be identified through the morphological features of the egg / cyst / oocyst or of the free-living stages of the parasite (Roberts, 1970, Barker *et al*., 1988, Spratt *et al*., 1991, Marquardt *et al*., 2000). Morphological identification thus depends heavily on knowledge of the parasite's life cycle.

Parasite species are further described based on host specificity, location inside the host, geographical distribution or pathogenicity (Duszynski & Wilber, 1997, Beveridge & Spratt, 2015). Recent molecular studies have greatly expanded upon current knowledge from morphological identifications and resulted in many taxonomic revisions (Xiao *et al*., 1999, Cacciò, 2004, Beveridge & Shamsi, 2009, Blake, 2015). Thorough genetic analyses have revealed a high diversity of cryptic parasite species, particularly in host specific parasites (Poulin & Keeney, 2008). For example, the number of species of the highly host specific and endemic helminth parasites of Australasian marsupials has expanded from 434 in 1991 (Spratt *et al*., 1991, Beveridge & Spratt, 1996) to 658 in 2015 (Beveridge & Spratt, 2015), with many cryptic species still undescribed as only molecular data is currently available (Beveridge & Gasser, 2014).

To create an up to date database of all recorded parasites in *Petrogale* spp., a literature review was conducted of all available scientific journal records and books. A literature search was conducted of original research papers in online databases, namely Scopus, ScienceDirect and Web of Knowledge, using the search terms '*Petrogale*', 'rock-wallaby' and / or 'rock-wallabies', with adjustments made due to revisions in taxonomy of some parasite species, as well as consulting reference records such as Spratt *et al*. (1991) and Roberts (1970).

2.4.1 Protozoa

Table 2.2: Identified protozoan parasites in rock-wallabies, *Petrogale*.

Parasite species	**Host species (*Petrogale*)**	**References**
Babesia sp.	*P. persephone*	O'Donoghue 1997
Cryptosporidium fayeri	*P. penicillata, P. xanthopus*	Power *et al*., 2003, Power *et al*., 2009a, Vermeulen *et al*., 2015a
Cryptosporidium meleagridis	*P. penicillata*	Vermeulen *et al*., 2015a
Eimeria boonderooensis	*P. assimilis, P. inornata, P. mareeba, P. sharmani*	Barker *et al*., 1988
Eimeria godmani	*P. assimilis, P. godmani, P. inornata, P. lateralis*	Barker *et al*., 1988, O'Callaghan *et al*., 1998, Duszynski, 2015
Eimeria inornata	*P. inornata, P. lateralis, P. penicillata*	Barker *et al*., 1988, O'Callaghan *et al*., 1998
Eimeria occidentalis	*P. lateralis, P. rothschildi, P. wilkinsi*	Barker *et al*., 1988
Eimeria petrogale	*P. assimilis, P. godmani, P. herberti, P. inornata, P. lateralis, P. mareeba, P. penicillata, P. persephone, P. sharmani*	Barker *et al*., 1988, Begg *et al*., 1995, O'Callaghan *et al*., 1998
Eimeria sharmani	*P. assimilis, P. godmani, P. herberti, P. inornata, P. lateralis, P. mareeba, P. penicillata, P. persephone, P. rothschildi, P. sharmani*	Barker *et al*., 1988, Begg *et al*., 1995, O'Callaghan *et al*., 1998
Eimeria xanthopus	*P. xanthopus*	Barker *et al*., 1988
Eimeria sp.	*P. herberti*	Barker *et al*., 1988
Giardia duodenalis, A and B	*P. penicillata, P. xanthopus*	Thompson *et al*., 2008, Vermeulen *et al*., 2015b
Sarcocystis mucosa	*P. assimilis, P. inornata, P. penicillata*	Levine 1979, O'Donoghue *et al*., 1987, Dubey *et al*., 1989, Duszyinski, 2015
Sarcocystis macropodis	*P. xanthopus*	Gilruth & Bull 1912
Sacrocystis sp.	*P. concinna, P. penicillata, P. wilkinsi*	Munday 1978, Munday *et al*., 1978, Speare *et al*., 1989
Toxoplasma gondii	*P. penicillata, P. xanthopus*	Dubey & Beattie 1988, Barnes *et al*., 2010
Trypanosoma theileri or *T. cyclops*	*P. penicillata*	Hamilton *et al*., 2005

2.4.1.1 Enteric protozoan parasites

Three genera of enteric protozoan parasites, *Eimeria*, *Cryptosporidium* and *Giardia*, have been identified in *Petrogale* (Table 2.2). *Giardia* is a flagellate protozoan that reproduces asexually through binary fission and colonises the gut lumen (Adam, 2001), while *Cryptosporidium* and *Eimeria* are obligatory intracellular coccidian parasites, which invade the gut epithelial cells and reproduce sexually through gametocytes (Marquardt *et al*., 2000).

All three genera have a direct faecal-oral transmission route via the infective stages present in the faeces (cysts / oocysts) (Marquardt *et al*., 2000). Records of these enteric protozoa in rock-wallabies are based on the presence and morphological analyses of their cysts / oocysts from the faeces (Barker *et al*., 1988). More recently, protozoan parasite records for some host wallaby species have included molecular analyses (Power *et al*., 2009, Thompson *et al*., 2010b, Hill *et al*., 2012, Vermeulen *et al*., 2015a = Chapter 3).

Eimeria (Apicomplexa: Eimeriidae) is a highly diverse coccidian parasite genus that currently contains more than 1,200 described species identified using morphological characters and host traits (Duszynski & Wilber, 1997, Chapman *et al*., 2013). Many *Eimeria* species are highly host-specific (Duszynski & Wilber, 1997), but host-sharing has been recorded (Barker *et al*., 1989, Zhao *et al*., 2001). Seven species of *Eimeria* have been identified in *Petrogale* hosts (Barker *et al*., 1988, Begg *et al*., 1995, O'Callaghan *et al*., 1998). Two *Eimeria* species, *E. petrogale* and *E. sharmani*, are found in six *Petrogale* species which are either closely related (within the *lateralis-penicillata* group) or have overlapping geographical ranges (Briscoe *et al*., 1982, Barker *et al*., 1988, Potter *et al*., 2012). Four *Eimeria* species have been described in two or three *Petrogale* species (Barker *et al*., 1988) and one species, *E. xanthopus*, is only found in *P. xanthopus* (Barker *et al*., 1988). There are no records of *Eimeria* causing disease in rock-wallaby hosts. However, this parasite can cause disease with severe diarrhoea and high morbidity in other animals under specific circumstances. Records of clinical coccidiosis in marsupials are limited to reports from captive bred or juveniles (Hum *et al*., 1991), or wild populations whose habitat was highly disturbed (Barker *et al*., 1972).

Cryptosporidium (Apicomplexa: Cryptosporidiidae) is a coccidian parasite genus containing species with narrow and broad host ranges (Ryan *et al*., 2014). Of the 27 currently recognized species (Ryan *et al*., 2014, 2015), two are most commonly identified in marsupials; namely

C. fayeri and *C. macropodum* (Power & Ryan, 2008, Ryan *et al*., 2008). *Cryptosporidium* has only been identified in two *Petrogale* species, *P. penicillata* and *P. xanthopus* (Table 2.2). The marsupial specific species *C. fayeri* was identified in both host species (Power *et al*., 2003, Power *et al*., 2009, Vermeulen *et al*., 2015a = Chapter 3). A species typically found in birds and human clinical cases, *C. meleagridis*, has been identified recently in *P. penicillata* (Vermeulen *et al*., 2015a).

Giardia duodenalis (Diplozoa: Giardiidae) is a protozoan parasite of diverse mammalian hosts. *Giardia duodenalis* is considered a species complex of genetically defined assemblages (A-H) and sub-assemblages (e.g. AI to AIV) which vary in host specificity and pathogenicity (Read *et al*., 2002, Cacciò & Ryan, 2008, Feng & Xiao, 2011). The zoonotic genotypes of *Giardia* commonly found in humans, assemblages A and B, have also been found in many wildlife species, including marsupials (McCarthy *et al*., 2008, Thompson *et al*., 2008, Thompson *et al*., 2010b, Ng *et al*., 2011). Only two species of *Petrogale* have been investigated for *G. duodenalis*, namely *P. penicillata* and *P. xanthopus* (Table 2.2). *Giardia* in these rock-wallaby hosts were assigned to the zoonotic assemblages A and B. While the infected *P. xanthopus* was a captive animal (Thompson *et al*., 2008), the *P. penicillata* was from a wild population (Vermeulen *et al*., 2015b = Chapter 4), indicating a transmission route for *G. duodenalis* between humans, domestic animals and wildlife.

2.4.1.2 Blood-borne and tissue protozoa

Four genera of tissue and blood-borne protozoan parasites, two of which are vector-borne, have been identified in *Petrogale* species (Table 2.2). As these parasites reside either in the tissue or blood, records of these parasites are made typically from morphological description through post-mortem examination (O'Donoghue *et al*., 1987), from blood smears (Donahoe *et al*., 2015) or by identifying antibodies to the parasite (Barnes *et al*., 2010).

Toxoplasma gondii (Apicomplexa: Sarcocystidae) is a coccidian parasite that forms tissue cysts in numerous body systems. The parasite is found in diverse vertebrate hosts; however, it has a facultative heteroxenous life cycle with Felidae (typically *Felis catus*) as the definitive host and their prey as the intermediate host (Tenter *et al*., 2000). *Toxoplasma gondii* can also be transmitted directly via ingestion of the infective oocyst excreted in cat faeces that can contaminate the environment or food, a pathway that has facilitated transmission to many vertebrate hosts, including marsupials (Patton *et al*., 1986, Pan *et al*., 2012). Infection can result in toxoplasmosis, which affects the central nervous system, can cause abortion or congenital defects, or death of the host, and has been observed in wild and captive macropodoids (Obendorf & Munday, 1983, Johnson *et al*., 1988, Tenter *et al*., 2000). Clinical symptoms have mainly been observed in captive populations (Thompson *et al*., 2009), but there is increasing evidence that *Toxoplasma* infection has a compounding negative impact on threatened marsupial populations when combined with the presence of introduced predators like cats, which increase the prevalence of *Toxoplasma* and induce stress on the host, and competition for food with introduced domestic animals (McCallum & Dobson, 2002, Abbott, 2006, Thompson *et al*., 2010a). To date, *T. gondii* has only been recorded in two *Petrogale* species, *P. penicillata* and *P. xanthopus* based on antibody detection and identification of tissue cysts respectively (Dubey & Beattie, 1988, Barnes *et al*., 2010).

Sarcocystis (Apicomplexa: Sarcocystidae) has an obligatory two host life cycle and resides in the muscle tissue of the intermediate host, typically the striated muscles (Dubey *et al*., 1989). Pre-cyst development stages (schizonts) can lead to severe disease by rupturing the blood vessels and the severity of the pathology is dependent on the number of sporocysts ingested (Chhabra & Samantaray, 2013). The genus has a wide host range mainly in mammals, which includes Australian marsupials (O'Donoghue *et al*., 1987, Chhabra & Samantaray, 2013). *Sarcocystis* species have been described in six *Petrogale* species (Table 2.2). One study

described the cysts that were predominantly found in the muscularis externa and submucosa of the gastrointestinal tract of the host (O'Donoghue *et al*., 1987).

Trypanosoma (Euglenozoa: Trypanosomatida) and *Babesia* (Apicomplexa: Babesiidae) are both vector-borne blood parasites with broad host ranges. *Trypanosoma* species cause a variety of severe diseases including sleeping sickness and Chagas disease in humans and can cause clinical signs similar to these diseases in various other animals, including macropodoids (Reid, 2002, Thompson, 2013). *Trypanosoma* species are typically spread through blood-feeding arthropods or leeches (Hamilton *et al*., 2005). Infection by introduced *Trypanosoma* species and subsequent Chagas-disease like symptoms have been linked to a sharp population decline in a threatened marsupial, the brush-tailed bettong (*Bettongia penicillata*) (Botero *et al*., 2013) and extinction of another indigenous Australian mammal, Maclear's rat (*Rattus macleari*) (Wyatt *et al*., 2008). These examples indicate that the presence of *Trypanosoma* species in threatened marsupials is of concern to conservation (Thompson, 2013). Similarly, *Babesia* species cause disease in macropodoids with anaemia as the typical clinical sign (Donahoe *et al*., 2015). *Babesia* are vector-borne parasites that are spread by ticks (Mehlhorn & Shein 1984). Clinical signs in macropodids caused by *Babesia* species such as anaemia have been observed only in captive kangaroos (Dawood *et al*. 2013), but too few studies have been conducted on *Babesia* in wildlife to fully understand the diversity and pathogenicity of this genus in wild macropodids (Thompson 2013). To date, *Trypanosoma* and *Babesia* species have only been described in one *Petrogale* species each, *P. penicillata* and *P. persephone* respectively (Table 2.2).

2.4.2 Helminths

2.4.2.1 Diversity of helminths recorded in the family Macropodidae and genus *Petrogale*

Macropodoids, including rock-wallabies, harbour a huge diversity of helminth species with over 658 species described to date (Beveridge & Spratt, 2015). A total of 12 helminth families comprising 31 genera and 110 species have been recorded in *Petrogale* hosts (Table 2.3). The highest diversity was observed in the sub-family Cloacininae with a total of 82 species recorded (Table 2.3). Within the Cloacininae, the genus *Cloacina* is the most diverse with 35 recorded species with a core group of species such as *C. caenis, C. ernabella, C. pearsoni* and *C. robertsi* being found only in *Petrogale* while many other species are shared with sympatric macropodoids (Gibson *et al*., 2005, Beveridge & Spratt, 2015). All helminth species described to date in *Petrogale* are endemic to macropodoids, with the exception of two introduced species, *Physocephalus sexalatus* and *Echinococcus granulosus* (Beveridge *et al*., 2010). As *Physocephalus sexalatus* is a spiruroid nematode typically found in pigs and wild boars, this parasite would have been introduced to the Australian environment with the introduction of swine (Foster, 1912, Gassó *et al*., 2015). The larval stage of the cestode *E. granulosus* (hydatid tapeworm) typically forms cysts in the lungs of domestic livestock, but has developed a sylvatic cycle in Australia between wild canids and macropodoids, including rock-wallabies (Barnes *et al*., 2007, 2008). There is some evidence of high host specificity in helminths, particularly in cloacinine nematodes, in *Petrogale*, with some parasite species only being found in a single *Petrogale* host species, namely two cloacinine species recorded only in *P. persephone*, three in *P. wilkinsi* and another three species only in *P. lateralis* (Spratt *et al*., 1991, Gibson *et al*., 2005). However, most of the helminth species have also been found in several other marsupial species and thus there appears to be host-sharing with sympatric species, particularly within macropodoids (Spratt *et al*., 1991, Gibson *et al*., 2005, Beveridge *et al*., 2010).

Table 2.3: Identified of helminth parasites in rock-wallabies, *Petrogale*, divided into Cestoda (Platyhelminthes) and Nematoda, with the order, superfamily and family for clarification of taxonomy. Parasites are ordered by genus name with each class.

Class	Order	Superfamily	Family/Subfamily	Tribe	Genus/species	Host species (*Petrogale*)	References
Cestoda	Cyclophyllidea		Taeniidae		*Echinococcus granulosus*	*P. assimilis, P. godmani, P. inornata, P. mareeba, P. penicillata, P. persephone*	Beveridge *et al*., 1989, Begg *et al*., 1995, Johnson *et al*., 1998, Spratt, 2002, Barnes *et al*., 2008
	Cyclophyllidea		Anoplocephalidae		*Progamotaenia capricorniensis*	*P. assimilis*	Beveridge & Turni 2003
	Cyclophyllidea		Anoplocephalidae		*Progamotaenia festiva*	*P. lateralis*	Beveridge & Shamsi 2009
	Cyclophyllidea		Anoplocephalidae		*Progamotaenia petrogale*	*P. assimilis, P. godmani, P. herberti, P. inornata, P. mareeba, P. persephone*	Beveridge, 2007
	Cyclophyllidea		Anoplocephalidae		*Progamotaenia* sp.	*P. lateralis*	Beveridge, 2007
	Cyclophyllidea		Anoplocephalidae		*Triplotaenia fimbriata*	*P. lateralis, P. purpureicollis*	Beveridge *et al*., 1989, Spratt *et al*., 1991
	Cyclophyllidea		Anoplocephalidae		*Triplotaenia mirabilis*	*P. assimilis, P. inornata, P. lateralis, P. penicillata* (zoo record), *P. purpureicollis*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Bradley, 2000, Hardman *et al*., 2012
	Cyclophyllidea		Anoplocephalidae		*Wallabicestus ewersi*	*P. assimilis, P. penicillata*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Hu *et al*., 2005
Nematoda	Strongylida	Strongyloidea	Cloacininae	Macropostrongylinea	*Alocostoma clelandi*	*P. penicillata*	Schultz *et al*., 2011
	Spirurida	Filarioidea	Onchocercidae		*Breinlia robertsi*	*P. lateralis*	Mackerras, 1962
	Spirurida	Filarioidea	Onchocercidae		*Breinlia spelaea*	*P. assimilis, P. herberti, P. inornata, P. mareeba, P. penicillata, P. persephone, P. sharmani*	Beveridge *et al*., 1989, Beveridge *et al*., 1992, Begg *et al*., 1995, Barnes *et al*., 2010
	Spirurida	Filarioidea	Onchocercidae		*Breinlia (Johnstonema) woerlei*	*P. concinna, P. wilkinsi*	Spratt & Varughese, 1975, Spratt *et al*., 1991, Spratt, 2011
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina australis*	*P. persephone*	Begg *et al*., 1995, Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina caenis*	*P. assimilis, P. brachyotis, P. coenensis, P. godmani, P. herberti, P. inornata, P. lateralis, P. mareeba, P. purpureicollis, P. sharmani, P. wilkinsi, P. xanthopus*	Beveridge, 1998a, Bradley, 2000, Chilton *et al*., 2009
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina cloelia*	*P. inornata, P. persephone*	Beveridge *et al*., 1992, Beveridge, 1998a, Chilton *et al*., 2009
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina communis*	*P. xanthopus*	Spratt *et al*., 1991, Beveridge, 1998a

Table 2.3 Continued

Class	Order	Superfamily	Family/Subfamily	Tribe	Genus/species	Host species (*Petrogale*)	References
Nematoda	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina curta*	*P. lateralis, P. xanthopus*	Spratt *et al*., 1991, Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina cybele*	*P. persephone*	Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina dahli*	*P. persephone*	Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina daveyi*	*P. xanthopus*	Spratt *et al*., 1991, Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina digitata*	*P. herberti, P. penicillata*	Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina echidne*	*P. purpureicollis*	Bradley, 2000
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina elegans*	*P. lateralis*	Spratt *et al*., 1991, Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina enyo*	*P. wilkinsi*	Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina ernabella*	*P. lateralis, P. purpureicollis*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Beveridge, 1998a, Bradley, 2000, Chilton *et al*., 2009
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina expansa*	*P. penicillata*	Schultz *et al*., 2011
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina feronia*	*P. assimilis, P. xanthopus*	Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina frequens*	*P. purpureicollis, P. xanthopus*	Spratt *et al*., 1991, Beveridge, 1998a, Bradley, 2000
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina herceus*	*P. assimilis, P. godmani, P. inornata, P. penicillata, P. sharmani*	Beveridge, 1998a, Schultz *et al*., 2011
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina hydriformis*	*P. lateralis, P. purpureicollis*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina io*	*P. assimilis*	Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina lityerses*	*P. lateralis*	Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina longelabiata*	*P. lateralis, P. purpureicollis, P. xanthopus*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Beveridge, 1998a, Bradley, 2000
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina macropodis*	*P. lateralis, P. purpureicollis, P. xanthopus*	Spratt *et al*., 1991, Beveridge, 1998a, Bradley, 2000
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina parva*	*P. lateralis, P. purpureicollis, P. wilkinsi, P. xanthopus*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Beveridge, 1998a, Bradley, 2000

Table 2.3 Continued

Class	Order	Superfamily	Family/Subfamily	Tribe	Genus/species	Host species (*Petrogale*)	References
Nematoda	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina pearsoni*	*P. assimilis, P. coenensis, P. godmani, P. herberti, P. inornata, P. lateralis, P. mareeba, P. penicillata, P. purpureicollis, P. wilkinsi, P. xanthopus*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Beveridge, 1998a, Bradley, 2000, Chilton *et al*., 2009
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina petrogale*	*P. lateralis, P. purpureicollis, P. wilkinsi*	Beveridge *el al*., 1989, Spratt *et al*., 1991, Beveridge, 1998a, Chilton *et al*., 1997a, Bradley, 2000
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina petronius*	*P. assimilis, P. coenensis, P. godmani, P. herberti, P. inornata, P. mareeba, P. sharmani*	Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina phaethon*	*P. assimilis, P. wilkinsi*	Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina robertsi*	*P. assimilis, P. coenensis, P. godmani, P. herberti, P. inornata, P. mareeba, P. penicillata, P. persephone, P. purpureicollis, P. sharmani, P. wilkinsi, P. xanthopus*	Beveridge, 1998a, Chilton *et al*., 2009
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina selene*	*P. penicillata*	Schultz *et al*., 2011
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina similis*	*P. assimilis, P. godmani, P. herberti, P. inornata, P. lateralis, P. penicillata, P. xanthopus*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Chilton *et al*., 1997a, Beveridge, 1998a
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina* sp.	*P. assimilis, P. penicillata, P. persephone*	Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina sp.*	*P. assimilis, P. herberti, P. inornata, P. mareeba, P. purpureicollis, P. sharmani*	Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina sp.*	*P. wilkinsi, P. xanthopus*	Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina sp.*	*P. persephone*	Begg *et al*., 1995
	Strongylida	Strongyloidea	Cloacininae	Cloacininea	*Cloacina* sp.	*P. lateralis, P. xanthopus*	Beveridge *et al*., 2002
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Coronostrongylus barkeri*	*P. wilkinsi*	Beveridge, 2002
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Coronostrongylus closei*	*P. persephone*	Beveridge, 2002
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Coronostrongylus coronatus*	*P. inornata*	Beveridge, 2002
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Coronostrongylus johnsoni*	*P. godmani, P. wilkinsi*	Beveridge, 2002

Table 2.3 Continued

Class	Order	Superfamily	Family/Subfamily	Tribe	Genus/species	Host species (*Petrogale*)	References
Nematoda	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Coronostrongylus sharmani*	*P. coenensis, P. godmani, P. mareeba*	Beveridge, 2002
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Coronostrongylus spratti*	*P. assimilis, P. inornata*	Beveridge, 2002
	Strongylida	Trichostrongyloidea	Dromaeostrongylidae		*Filarinema australe*	*P. assimilis, P. inornata, P. sharmani*	Beveridge & Spratt, 1988, Beveridge *et al*., 1989
	Strongylida	Trichostrongyloidea	Dromaeostrongylidae		*Filarinema dissimile*	*P. assimilis*	Beveridge & Spratt, 1988, Beveridge *et al*., 1989
	Strongylida	Trichostrongyloidea	Dromaeostrongylidae		*Filarinema mawsonae*	*P. assimilis, P. godmani, P. herberti, P. mareeba, P. penicillata, P. sharmani*	Beveridge & Spratt, 1988, Beveridge *et al*., 1989, Spratt *et al*., 1991
	Strongylida	Trichostrongyloidea	Globocephaloidinae		*Globocephaloides macropodis*	*P. assimilis, P. godmani, P. herberti, P. inornata, P. persephone, P. sharmani, P. wilkinsi*	Beveridge *et al*., 1989, Begg *et al*., 1995, Fazenda *et al*., 2010
	Strongylida	Trichostrongyloidea	Globocephaloidinae		*Globocephaloides wallabiae*	*P. assimilis, P. mareeba*	Fazenda *et al*., 2010
	Strongylida	Strongyloidea	Phascolostrongylinae	Hypodontinea	*Hypodontus macropi*	*P. persephone*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Begg *et al*., 1995, Chilton *et al*., 1995, Chilton *et al*., 1997b, Gasser *et al*., 2001, Chilton *et al*., 2012
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiomultiplex nabarlekensis*	*P. wilkinsi*	Smales, 1994
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex australis*	*P. lateralis*	Smales, 1995
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex bancrofti*	*P. assimilis, P. herberti, P. inornata, P. sharmani*	Beveridge *et al*., 1989, Spratt *et al*., 1991, Smales, 1995
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex centralis*	*P. lateralis*	Smales, 2006
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex flanneryi*	*P. assimilis*	Smales, 1995
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex godmani*	*P. coenensis, P. godmani*	Smales, 1995
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex longispicularis*	*P. xanthopus*	Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex pearsonensis*	*P. lateralis*	Smales, 1995
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex petrogale*	*P. lateralis*	Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiosimplex* sp.	*P. inornata, P. lateralis*	Spratt *et al*., 1991, Smales, 1995

Table 2.3 Continued

Class	Order	Superfamily	Family/Subfamily	Tribe	Genus/species	Host species (*Petrogale*)	References
Nematoda	Strongylida	Strongyloidea	Cloacininae	Labiostrongylinea	*Labiostrongylus labiostrongylus*	*P. wilkinsi*	Smales, 1994
	Strongylida	Strongyloidea	Phascolostrongylinae	Macropostrongyloidinea	*Macropostrongyloides baylisi*	*P. purpureicollis, P. xanthopus*	Spratt *et al*., 1991, Beveridge *et al*., 1993, Bradley, 2000
	Strongylida	Strongyloidea	Cloacininae	Macropostrongylinea	*Macropostrongylus macropostrongylus*	*P. godmani*	Beveridge, 1985, Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Macropostrongylinea	*Macropostrongylus petrogale*	*P. assimilis, P. godmani*	Beveridge, 1985, Beveridge *et al*., 1989, Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Macropostrongylinea	*Macrostrongylus sp.*	*P. wilkinsi*	Spratt *et al*., 1991
	Oxyurida	Oxyuroidea	Oxyuridae		*Macropoxyuris* sp.	*P. assimilis, P. godmani, P. herberti, P. inornata*	Beveridge *et al*., 1989
	Oxyurida	Oxyuroidea	Oxyuridae		*Macropoxyuris* sp.	*P. assimilis, P. godmani, P. herberti, P. inornata, P. penicillata, P. wilkinsi*	Spratt *et al*., 1991
	Oxyurida	Oxyuroidea	Oxyuridae		*Macropoxyuris* sp.	*P. persephone*	Begg *et al*., 1995
	Oxyurida	Oxyuroidea	Oxyuridae		*Macropoxyuris* sp.	*P. purpureicollis*	Bradley, 2000
	Ascaridida	Ascaridoidea	Ascarididae		*Ophidascaris robertsi (formerly Dipetalonema sp.)*	*P. inornata, P. penicillata*	Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Papillostrongylus barbatus*	*P. assimilis, P. purpureicollis*	Chilton *et al*., 2002
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Papillostrongylus labiatus*	*P. wilkinsi*	Beveridge *et al*., 1989, Chilton *et al*., 2002
	Spirurida	Filarioidea	Onchocercidae		*Pelecitus roemeri*	*P. assimilis, P. inornata, P. persephone*	Beveridge *et al*., 1989, Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Pharyngostrongylus kappa*	*P. assimilis*	Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Pharyngostrongylus lambda*	*P. assimilis, P. herberti, P. purpureicollis*	Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Pharyngostrongylus macropodis*	*P. assimilis, P. godmani, P. inornata, P. sharmani, P. wilkinsi*	Yorke & Maplestone, 1926, Beveridge, 1982, Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Pharyngostrongylus nelsoni*	*P. wilkinsi*	Beveridge, 1982, Spratt *et al*., 1991
	Spirurida	Spiruroidea	Spirocercidae	Spirocercinae	*Physocephalus sexalatus*	*P. assimilis*	Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Popovastrongylus pearsoni*	*P. lateralis*	Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Popovastrongylus thylogale*	*P. persephone*	Beveridge, 1986, Beveridge *et al*., 1989, Spratt *et al*., 1991, Begg *et al*., 1995
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx alpha*	*P. lateralis, P. purpureicollis*	Beveridge & Chilton, 1999, Bradley, 2000

Table 2.3 Continued

Class	Order	Superfamily	Family/Subfamily	Tribe	Genus/species	Host species (*Petrogale*)	References
Nematoda	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx australis*	*P. xanthopus*	Beveridge, 1982, Beveridge & Chilton, 1999
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx longibursaris*	*P. penicillata*	Schultz *et al*., 2011
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx petrogale*	*P. herberti, P. inornata, P. penicillata*	Beveridge & Chilton, 1999
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx rho*	*P. lateralis*	Beveridge & Chilton, 1999
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx sigma*	*P. persephone*	Begg *et al*., 1995
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx zeta*	*P. assimilis, P. herberti, P. inornata, P. mareeba, P. penicillata, P. sharmani*	Beveridge *et al*., 1989, Beveridge *et al*., 1994, Beveridge *et al*., 1995
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx* sp.	*P. persephone*	Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Rugopharynx* sp.	*P. lateralis*	Beveridge & Chilton, 1999
	Rhabditida	Rhabditoidea	Strongyloididae		*Strongyloides* sp.	*P. inornata*	Spratt *et al*., 1991
	Strongylida	Trichostrongyloidea	Herpetostrongylinae		*Sutarostrongylus petrogale*	*P. assimilis, P. godmani, P. herberti, P. mareeba, P. sharmani*	Beveridge & Durette-Desset, 1986, Beveridge *et al*., 1989
	Strongylida	Trichostrongyloidea	Herpetostrongylinae		*Sutarostrongylus safestatus*	*P. inornata, P. persephone*	Beveridge & Durette-Desset, 1986, Beveridge et al., 1989
	Strongylida	Strongyloidea	Cloacininae	Zoniolaiminea	*Thallostonema queenslandensis*	*P. persephone*	Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Coronostrongylinea	*Thylonema barkeri*	*P. persephone*	Beveridge *et al*., 1989
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Thylostrongylus* sp.	*P. inornata, P. penicillata*	Beveridge *et al*., 1989, Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Zoniolaiminea	*Wallabinema labiatum*	*P. penicillata*	Beveridge *et al*., 1989, Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Zoniolaiminea	*Wallabinema thylogale*	*P. persephone*	Begg *et al*., 1995
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Woodwardostrongylus obendorfi*	*P. assimilis, P. godmani, P. herberti, P. inornata, P. mareeba, P. penicillata, P. sharmani*	Beveridge *et al*., 1989, Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Woodwardostrongylus petrogale*	*P. wilkinsi*	Beveridge, 1998b
	Strongylida	Strongyloidea	Cloacininae	Pharyngostrongylinea	*Woodwardostrongylus woodwardi*	*P. lateralis*	Spratt *et al*., 1991
	Strongylida	Strongyloidea	Cloacininae	Zoniolaiminea	*Zoniolaimus petrogale*	*P. godmani, P. persephone*	Beveridge *et al*., 1989, Begg *et al*., 1995

2.4.2.2 Helminth parasites in rock-wallabies: location of parasites within host body and transmission patterns

The largest group of helminths found in *Petrogale*, the Cloacininae nematodes, occur in the sacculated forestomach and oesophagus (Beveridge & Chilton, 2001). Other parasites of the gastrointestinal tract include the oxyuroid genus *Macropoxyuris* in the colon (Beveridge *et al*., 1989), the strongyloid genera *Hypodontus and Macropostrongyloides* found in the caecum and colon and the trichostrongyloid genus *Sutarostrongylus* found in the duodenum (Beveridge *et al*., 1989, 2010, Beveridge & Chilton, 2001, Chilton *et al*., 2012). The anoplocephalid cestodes are typically found in the bile ducts or in the small intestine (Beveridge *et al*., 2010). Gastrointestinal tract nematodes usually have direct faecal-oral route life cycles (Anderson, 2000); however, there is evidence that the anoplocephalid cestodes may be transmitted through oribatid mites (Stunkard, 1969). The filarioid nematodes (*Breinlia* and *Pelecitus*) are found in various connective and intermuscular tissues, as well as blood vessels and subcutaneous tissue (Beveridge *et al*., 2010, Spratt, 2011). These nematodes are transmitted through intermediate arthropod hosts including tabanid flies and mosquitoes (Spratt, 1972, Yen, 1983).

2.4.3 Ectoparasites

Eleven genera of ectoparasites comprising 27 described species have been identified in rock-wallabies (Table 2.4). Macropodoids, including rock-wallabies, are typically infected by boopid lice (Phthiraptera: Boopiidae), which are highly speciose and also commonly infect various carnivorous marsupials, wombats and bandicoots (Murray & Calaby, 1971, Barker & Close, 1990). Twelve of the louse species that typically infect rock-wallabies belong to the genus *Heterodoxus* (Table 2.4) and have been found to be highly specific to rock-wallabies; however, significant host switching events have been documented between rock-wallaby

species with parapatric distributions (Barker, 1991). One record of another louse species, *Boopia notafusca*, was made in a population of *P. penicillata* introduced to New Zealand (NZ), this parasite may have come from contact with other animals in the NZ habitat (Palma, 1996). The only flea species recorded to date in *Petrogale*, *Leptosylla segnis* (Siphonaptera: Leptopsyllidae), usually occurs on introduced rodents, but also has been recorded from Australian native rodents and four marsupial species, including *P. xanthopus* (Hopkins & Rothschild, 1971, Dunnet & Mardon 1974). Among flies (Diptera: Hippoboscoidea), two species of the genus, *Ortholfersia*, have been described (Schultz *et al*., 2011). For the mites (Arachnida: Acari), species from five different genera have been reported (Table 2.4). One mite species, *Thadeua serrata*, has been reported in *P. persephone* to cause crateriform lesions on the inguinal and axillary skin (Skerratt *et al*., 2007).

Table 2.4: Identified ectoparasites in rock-wallabies, *Petrogale*.

Group name (common)	Parasite species	Host species (*Petrogale*)	References
Lice	*Boopia notafusca*	*P. penicillata*	Palma, 1996
	Heterodoxus ampullatus	*P. penicillata*	Barker & Close, 1990, Barker *et al*., 1992, Palma, 1996
	Heterodoxus briscoei	*P. herberti*	Barker & Close, 1990, Barker, 1991
	Heterodoxus closei	*P. assimilis*	Barker & Close, 1990, Barker, 1991
	Heterodoxus harrisoni	*P. assimilis*, *P. mareeba*	Barker & Close, 1990, Barker, 1991
	Heterodoxus hughendensis	*P. assimilis*	Barker & Close, 1990, Barker, 1991
	Heterodoxus insularis	*P. assimilis*, *P. inornata*, *P. mareeba*, *P. sharmani*	Barker, 1991, Barker *et al*., 1991, Barker *et al*., 1992
	Heterodoxus insulatus	*P. assimilis*	Clay, 1981, Barker & Close, 1990, Barker, 1991
	Heterodoxus lesouefi	*P. assimilis*	Barker & Close, 1990, Barker, 1991
	Heterodoxus maynesi	*P. herberti*, *P. inornata*	Barker & Close, 1990, Barker, 1991, Barker *et al*., 1992, Barker, 1996
	Heterodoxus murrayi	*P. coenensis*, *P. godmani*, *P. mareeba*	Barker & Close, 1990, Barker, 1991, Barker *et al*., 1992
	Heterodoxus octoseriatus	*P. herberti*, *P. inornata*, *P. penicillata*	Barker & Close, 1990, Barker, 1991, Barker *et al*., 1991, Barker *et al*., 1992, Barnes *et al*., 2010
	Heterodoxus orarius	*P. coenensis*, *P. godmani*	Barker & Close, 1990, Barker, 1991, Barker *et al*., 1992, Barker, 1996
	Heterodoxus sp.	*P. persephone*	Begg *et al*., 1995
	Heterodoxus sp.	*P. purpureicollis*	Barker, 1991
Fleas	*Leptopsylla segnis*	*P. xanthopus*	Hopkins & Rothschild, 1971
Hippoboscid flies	*Ortholfersia macleayi*	*P. penicillata*	Schultz *et al*., 2011
	Ortholfersia phaneroneura	*P. penicillata*	Schultz *et al*., 2011
Ticks	*Haemaphysalis bancrofti*	*P. penicillata*	Roberts, 1970, Barnes *et al*., 2010
	Haemaphysalis lagostrophi	*P. lateralis*	Roberts, 1970
	Haemaphysalis petrogalis	*P. inornata*, *P. persephone*	Roberts, 1970, Begg *et al*., 1995
	Ixodes hirsti	*P. penicillata*	Schultz *et al*., 2011
	Ixodes holocyclus	*P. penicillata*	Barnes *et al*., 2010
	Ixodes tasmani	*P. lateralis*	Roberts, 1970
Mite	*Guntheria (Derrickiella) queenslandia*	*P. penicillata*	Barnes *et al*., 2010
	Odontacarus adelaideae	*P. xanthopus*	O'Callaghan *et al*., 1994
	Petrogalochirus tasmaniensis	*P. penicillata*	Domrow, 1992
	Thadeua serrata	*P. persephone*	Begg *et al*., 1995, Skerratt *et al*., 2007
	Thadeua sp.	*P. penicillata*	Barnes *et al*., 2010
	Trombicula southcotti	*P. wilkinsi*	Domrow & Lester, 1985

Two genera of ticks (Arachnida: Acari) have been reported from skin examinations of *Petrogale* species: *Ixodes* and *Haemaphysalis* (Roberts, 1970, Begg *et al*., 1995, Barnes *et al*., 2010). Ticks occur most frequently around the ears and facial area and less frequently over the rest of the body (Barnes *et al*., 2010). Some *P. penicillata* infected with *Ixodes holocyclus* displayed granulomas, particularly with engorged female ticks, and mild dermatitis (Barnes *et al*., 2010). Tick paralysis is also caused by *I. holocyclus*, due to the toxins in the female's saliva (Eppleston *et al*., 2013). Rare cases of tick paralysis have been reported in marsupials, but none have been reported in *Petrogale* (Roberts, 1970, Barnes *et al*., 2010). Ectoparasites present further risks as many species of ticks, fleas or flies, are vectors for protozoan parasites such as *Trypanosoma* and *Babesia* and filarioid worms that may infect rock-wallabies (reviewed in Thompson *et al*., 2010a).

2.5 Host specificity of *Petrogale* parasites

Many parasite species are highly host specific (Joyner, 1982) and highly specialized parasites are at higher risk of coextinction with their threatened hosts (Koh *et al*., 2004a, Dunn *et al*., 2009). For species of *Petrogale*, it is important to assess whether the parasite communities of these diverse wallabies are highly host specific and whether those parasites associated with threatened hosts are at risk of coextinction? Furthermore, what aspects of the host's biology have affected the biodiversity and host specificity of their parasite assemblages?

2.5.1 Host specificity from a host phylogeny perspective

We hypothesised that if the parasites of rock-wallabies are highly host specific, a positive correlation between the dissimilarity of the parasite assemblages of each host species of *Petrogale* and the corresponding evolutionary distance between these *Petrogale* species would be observed. A strong correlation would indicate that *Petrogale* parasites are clade specific and thus highly host specific. Correlation models between phylogenetic relationships

and assemblages of associated dependent species have previously been assessed in host-parasite relationships (Krasnov *et al*., 2010, Poulin, 2010) and plant-herbivore beetle relationships (Nipperess *et al*., 2012).

To test for correlation between *Petrogale* phylogeny and the dissimilarity of their associated parasite assemblages, R v. 3.2.2 (R Core Team, 2015) was used for the statistical analyses and to generate the resulting graphs. To initially examine the specificity of parasites in *Petrogale*, a dissimilarity matrix was created using Sorenson's index of similarity. Sorenson's index compares the number of parasite species between a pair of *Petrogale* species and the number of parasite species shared by that pair to create a quotient of similarity that ranges between 0 and 1 (Magurran, 1988). The dissimilarity for each *Petrogale* host pair was calculated based on presence or absence of identification of a parasite species for each species of rock-wallaby. A non-metric multidimensional scaling ordination graph (MDS) was then created (Fig. 2.1) where distance between *Petrogale* species is indicative of the dissimilarity of their corresponding parasite assemblages. All *Petrogale* species were included except for *P. burbidgei* for which no records of any parasites are available. The overall trend of the ordination graph was an overlap of parasite community compositions within closely related *Petrogale* species (Fig. 2.1, Suppl. Fig. 2.1). The high distinctiveness of three of the outliers, *P. brachyotis*, *P. concinna* and *P. rothschildi*, is most likely due to the paucity of parasite records (only 1-2 species for each host) (Table 2.1-4).

To test for correlation between parasite dissimilarity and host phylogeny, evolutionary distance was measured as the age of the most recent common ancestor (MRCA, units: million years, Ma) estimated from the phylogram tree using both nuclear and mitochondrial DNA of 13 out of 17 *Petrogale* species from a previous study (Potter *et al*., 2012) (Suppl. Fig. 2.1). The evolutionary distance was then related to dissimilarity of parasites between each *Petrogale* pair, calculated through Sorenson's Index of Similarity (Magurran, 1988),

represented for each parasite group and all parasites together. We used a Mantel test to test for an association between parasite dissimilarity and MRCA using the Spearman correlation coefficient (Fig. 2.2) with the *p*-value calculated through 999 permutations of the data.

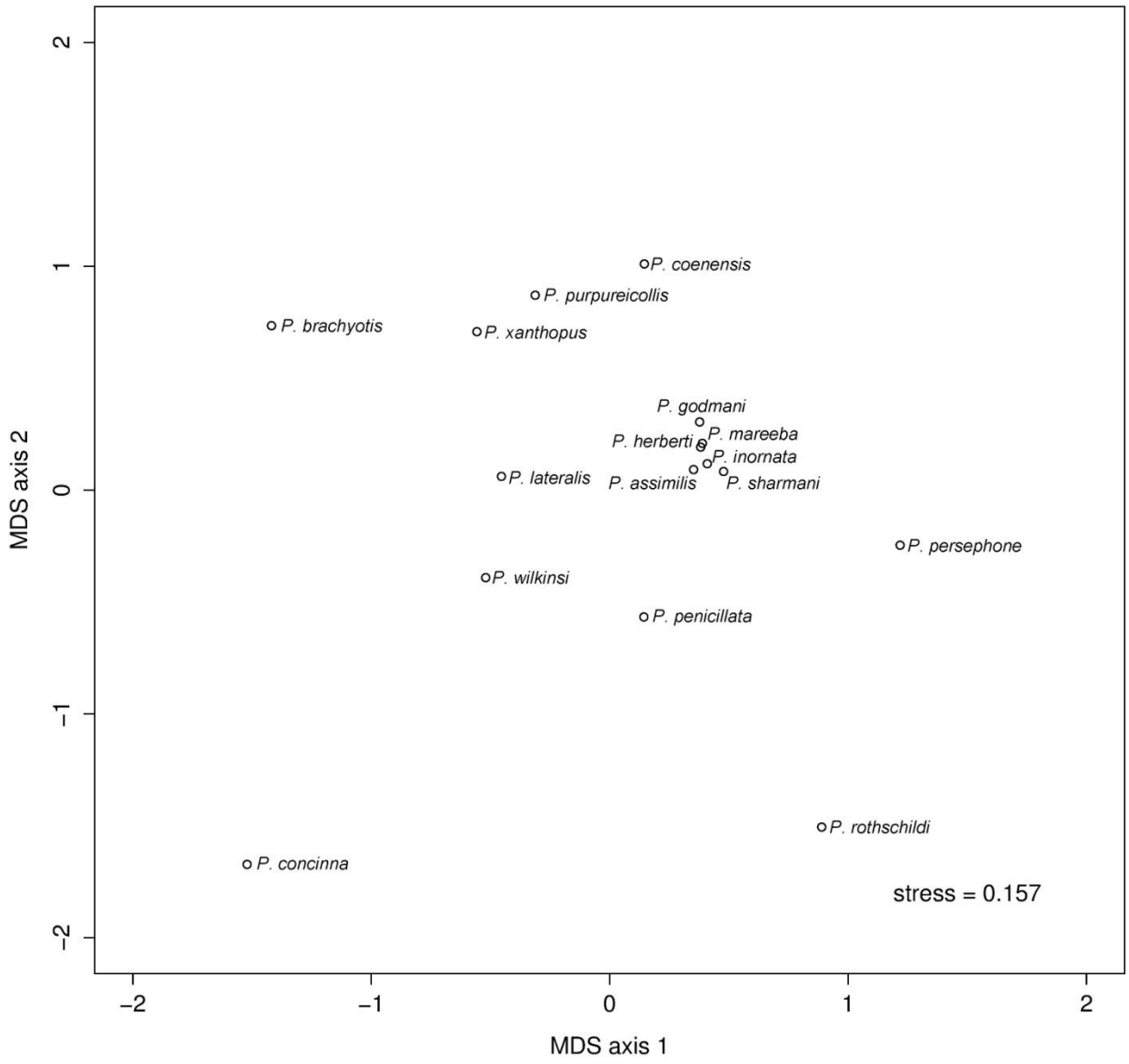


Figure 2.1: Non-metric multi-dimensional scaling (MDS) ordination, showing similarity of parasite assemblages between *Petrogale* hosts, based on the Sorenson's index of similarity. The ordination stress is represented on the graph.

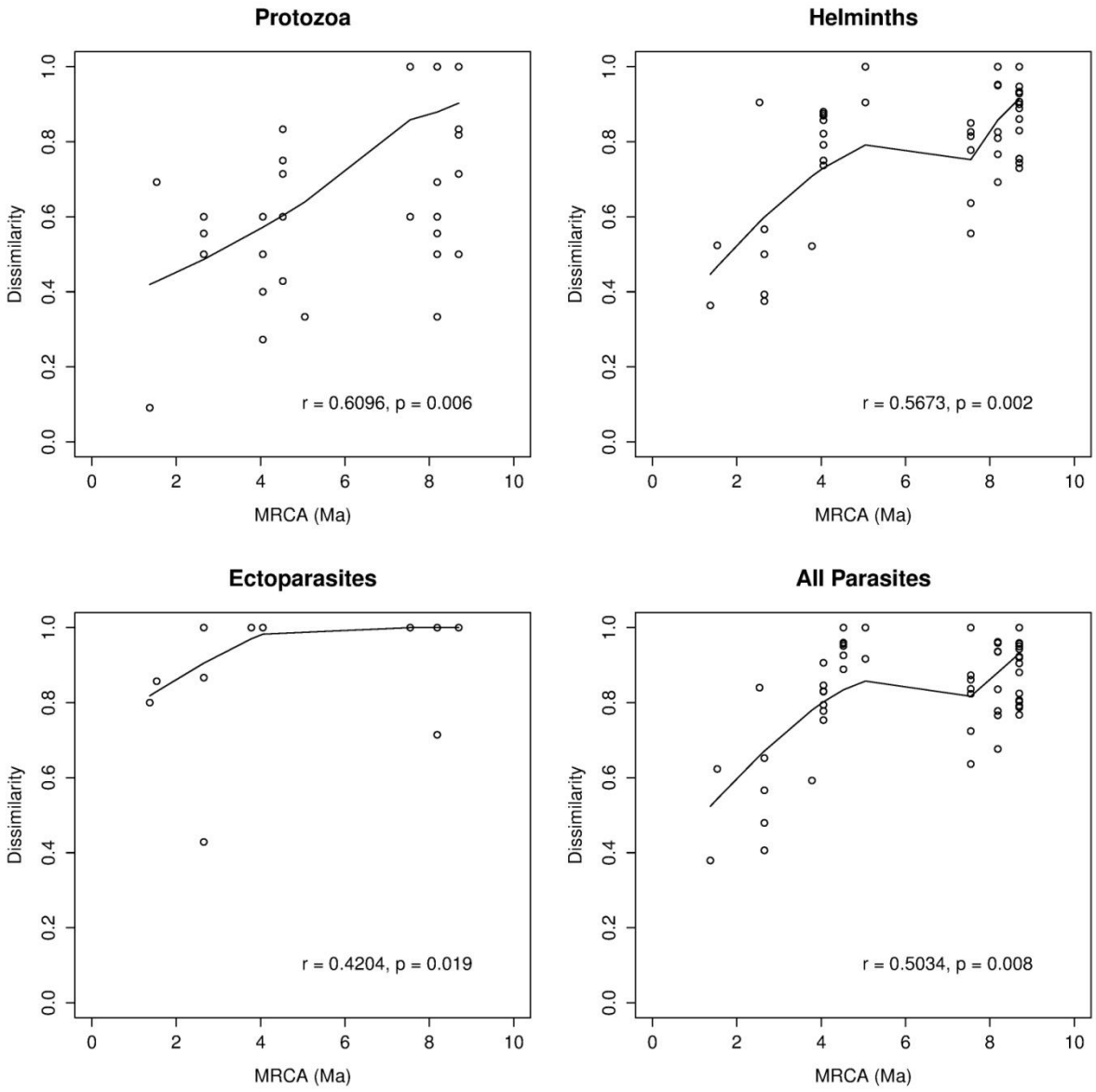


Figure 2.2: Scatter plots comparing evolutionary distance amongst 13 *Petrogale* hosts and dissimilarity of parasite assemblages for the different parasite groups: Protozoa, Helminths, Ectoparasites and All Parasites combined. MRCA denotes evolutionary distance as the age of the most recent common ancestor (Ma, million years) estimated from a recent phylogeny (Potter *et al*. 2012). A smoothed line (locally weighted regression) is fitted for illustration purposes only. The model was tested with the Mantel test using the Spearman correlation coefficient, with the Mantel statistic (r) and *p*-value represented on each graph.

For all four parasite categories (Protozoa, Helminths, Ectoparasites and All Parasites), a significant correlation was observed between parasite dissimilarity and MRCA. The most significant correlation was observed in internal parasites, particularly for helminths (r = 0.5673, p = 0.002). The strong correlation for helminths may be in part due to a much larger sample size for these parasites across *Petrogale* species, though a previous study has shown that only a low number of rock-wallabies need to be sampled to identify core species while further sampling may identify more host switches (Beveridge *et al*., 1989). Furthermore, helminth species are highly diverse and specialized within marsupials, particularly the cloacinine nematodes (Table 2.3, Beveridge & Spratt, 2015). Overall, there was a highly significant correlation for all parasites (r = 0.5034, p = 0.008), suggesting that host evolutionary relationships were an important contributor to the pattern of host-parasite community structure (Fig. 2.1).

2.5.2 Host specificity from a biogeographical perspective

A comparison of the ordination graph and distribution of *Petrogale* species showed an overall trend of similarity of parasite communities between host species whose distributions abut, particularly on the east coast of Australia (Fig. 2.1, Supp. Fig. 2.2). Previous studies on host-parasite associations have found a correlation between geographical distance of the host distributions and their parasite assemblages (Thompson, 1999, Poulin, 2003, Poulin *et al*., 2012). This biogeographical correlation may be due to the limitation of the distribution of host specific parasites to the range of their host species (Froeschke *et al*. 2013). The parasite distribution may be further limited by environmental conditions for transfer between hosts such as the presence of a vector or body of water (Thompson 1999). To test if *Petrogale* species whose distributions abut or overlap (parapatric) have more similar parasite assemblages than those whose distributions do not (allopatric), we defined each wallaby pair as either parapatric or allopatric defined by whether their distributions abut or not (i.e. have

adjoining or overlapping boundaries – see Suppl. Fig. 2.2) and compared the parasite dissimilarity of each pair between parapatric and allopatric wallabies (Fig. 2.3). The mean (± standard deviation) parasite dissimilarity was higher for the allopatric wallaby pairs (0.83 ± 0.15) than for the parapatric wallaby pairs (0.53 ± 0.20). The difference in means of parasite dissimilarity between allopatric and parapatric rock-wallaby pairs was tested for statistical significance through a non-parametric permutation test using 10,000 permutations; the resulting *p*-value was estimated to be 0.0001, and thus highly significant.

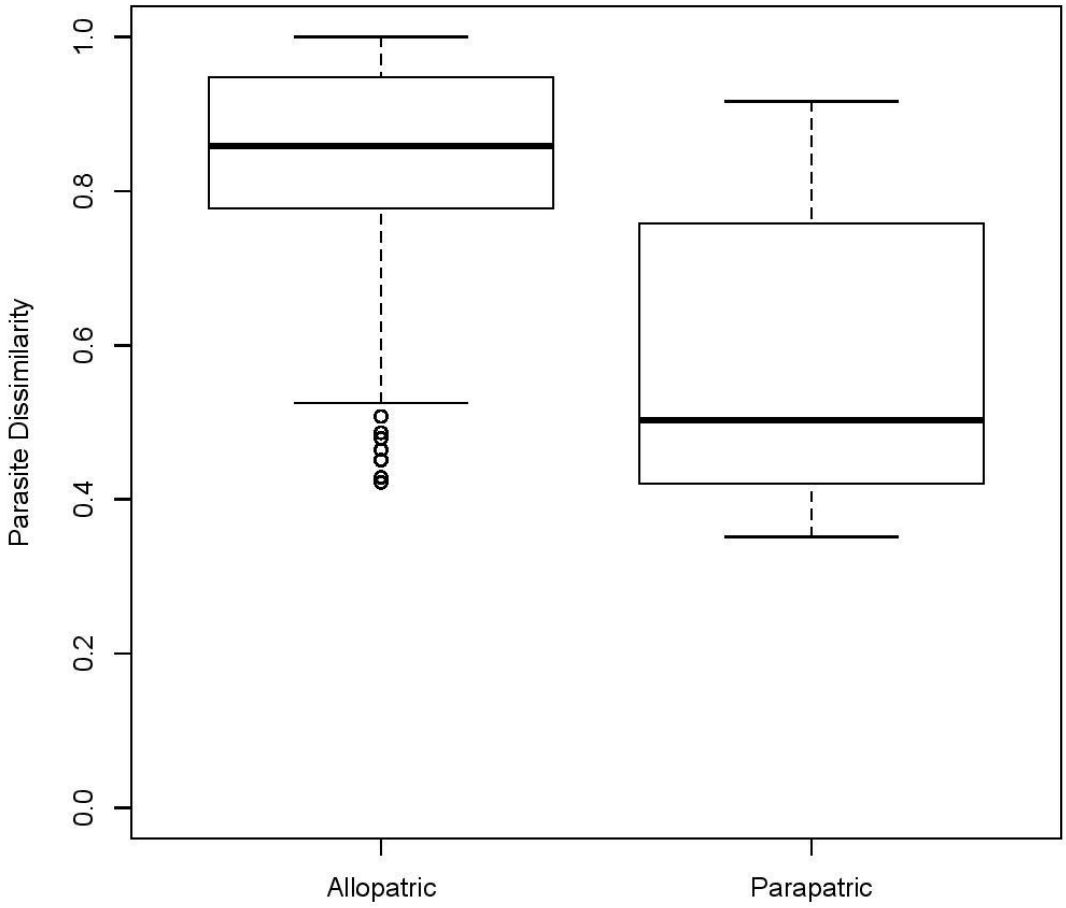


Figure 2.3: Box-and-whisker plot of the parasite dissimilarity comparing allopatric and parapatric pairs of *Petrogale* species.

Parapatric rock-wallabies may have a more similar parasite assemblage due to a higher chance for host sharing of parasites given host contact in abutting ranges (Eldridge & Close, 1993, Potter *et al*., 2012). Furthermore, some *Petrogale* species with parapatric distributions have only recently diverged and thus there may also be retention of ancestral parasite communities in these hosts (Eldridge & Close, 1993, Eldridge, 2008). Climatic factors may also have an influence on parasite distribution, as evidenced by the high dissimilarity of the parasite community of *P. penicillata* (Fig. 2.1), the only *Petrogale* species occurring in the temperate and mesic south-east of Australia (Eldridge & Close, 2005). However, the dissimilarity of the parasite assemblage of *P. penicillata* compared to closely related rock-wallabies (Supp. Fig. 2.1), may also be due to sampling bias. Many of the parasites unique to *P. penicillata* occurred in captive bred animals released into the wild as part of a reintroduction programs (Schultz *et al*., 2006, Taggart *et al*., 2008, 2009). These populations of rock-wallabies may have acquired parasites (mainly nematodes) from sympatric macropodids such as *Macropus rufogriseus*, *M. giganteus*, and *Wallabia bicolor* (Spratt *et al*., 1991, Schultz *et al*., 2011).

Climate and habitat may also play a role in the dissimilarity of *P. persephone.* Although its distribution falls within that of *P. inornata* (Suppl. Fig. 2.2), *P. persephone* is the only rock-wallaby that lives in rainforest (Maynes, 1982). However, many of the parasites unique to *P. persephone* compared to other rock-wallabies were also discovered in another sympatric macropodid, the red-legged pademelon *Thylogale stigmatica* (Begg *et al*., 1995) (Suppl. Fig. 2.1). Thus, the transfer of parasites between sympatric, related hosts may have contributed to the unique parasite assemblage of *P. persephone* within *Petrogale*.

Our analyses of *Petrogale* species suggest there is a complex interaction between biogeography, environmental conditions, host phylogeny and the presence of related sympatric hosts which all contribute to the community structure of parasites.

2.5.3 Comparison of the contribution of host phylogenetic and biogeographical effects on host specificity

As host phylogeny and biogeography form a complex interaction with each other (Nieberding *et al*., 2008, Krasnov *et al*., 2010, Potter *et al*., 2012), we aimed to untangle the confounding effects of each variable on parasite dissimilarity between rock-wallaby pairs. For those pairwise comparisons for which we had parasite, phylogenetic and geographical information (12 out of 17 rock-wallaby species), we did a multiple regression on the distance matrices. Following the procedure of Krasnov *et al*. (2010), we calculated the variance in parasite dissimilarity explained by host phylogeny (as MRCA) and biogeography (allopatry versus parapatry). The biogeographic distance matrix was coded as 1 for allopatric wallaby pairs and 0 for parapatric pairs. Distances were converted to ranks and significance was tested by 1000 permutations of the data. The explained variance of the model was partitioned into fractions corresponding to that solely explained by host phylogeny, solely explained by biogeography and that fraction jointly explained by host phylogeny and biogeography. This was done by fitting separate models for each of host phylogeny and biogeography, and subtracting the resulting explained variance from the combined model (Krasnov *et al*., 2010, Nipperess *et al*., 2012). This model was created using R v. 3.2.2 (R Core Team, 2015).

Of the total variance explained in the model ($R^2 = 0.270$, $F = 11.650$, $p = 0.009$), the effect of host phylogeny alone ($R^2 = 0.174$) was much greater than the effect of biogeography alone ($R^2 = 0.017$), with a substantial degree of overlap between the two ($R^2 = 0.080$). Based on available data, the dissimilarity of parasite assemblages between the pairs of rock-wallaby species is mostly explained by host phylogeny, with only a small additional contribution from biogeography. As we are lacking phylogenetic information on some parapatric rock-wallaby species pairs however, the results should be considered provisional until more phylogenetic information on the rock-wallabies becomes available.

However, given the strong effect of phylogeny, the data suggest that the parasite assemblages show strong fidelity to the specific host clades beyond that expected from geographical proximity. Therefore, the biodiversity of host specific parasite communities may be highly dependent on the genetic diversity of their hosts.

2.6 Discussion

2.6.1 Summary for parasite records in *Petrogale* and future directions

A total of 157 species of parasites has been described for 16 of the 17 *Petrogale* species, demonstrating a highly diverse parasite assemblage within *Petrogale*. The majority of records are for helminths, which comprise 70% (110/157) of parasite species records to date due to the efforts of parasitologists in Australia investigating this taxonomically diverse parasite group in marsupials (Beveridge & Spratt, 2015). Anthropozoonotic pathogens such as *Trypanosoma*, *Cryptosporidium*, *Giardia duodenalis* and *Echinococcus granulosus* have gained more focus recently due to concerns for wallabies as potential wildlife reservoirs (Thompson, 2000, 2013, Barnes *et al*., 2008, Thompson *et al*., 2010a, Ng *et al*., 2011, Vermeulen *et al*., 2015a, b = Chapters 3 and 4). Studying the parasite communities of rock-wallabies is limited by access to their steep and rocky habitats and inability to collect hosts due to their conservation status. Parasite records have mainly focused on four *Petrogale* species, *P. assimilis* (13% of total records for *Petrogale*), *P. inornata* (11%), *P. lateralis* (9%) and *P. penicillata* (12%). Of these species, the latter two are considered near threatened (IUCN, 2015) and all four species have large distributions relatively close to urban centres and research institutions (Eldridge, 2011, Potter *et al*., 2012). In contrast, *P. burbidgei* has no parasite records to date despite its near threatened status (IUCN, 2015). Its habitat is restricted to a small region in the remote Kimberley region of Western Australia (Eldridge & Close, 1993, Potter *et al*., 2012). This study has indicated that the parasite communities of

Petrogale are highly diverse, unique and host specific. However, future studies are required to fully document the diversity of parasites in this genus, particularly focusing on those *Petrogale* species where knowledge is lacking, such as the threatened *P. burbidgei*, or is rudimentary (e.g. *P. concinna*, *P. brachyotis* and *P. rothschildi*) as well as refine our knowledge on the phylogeny of rock-wallabies.

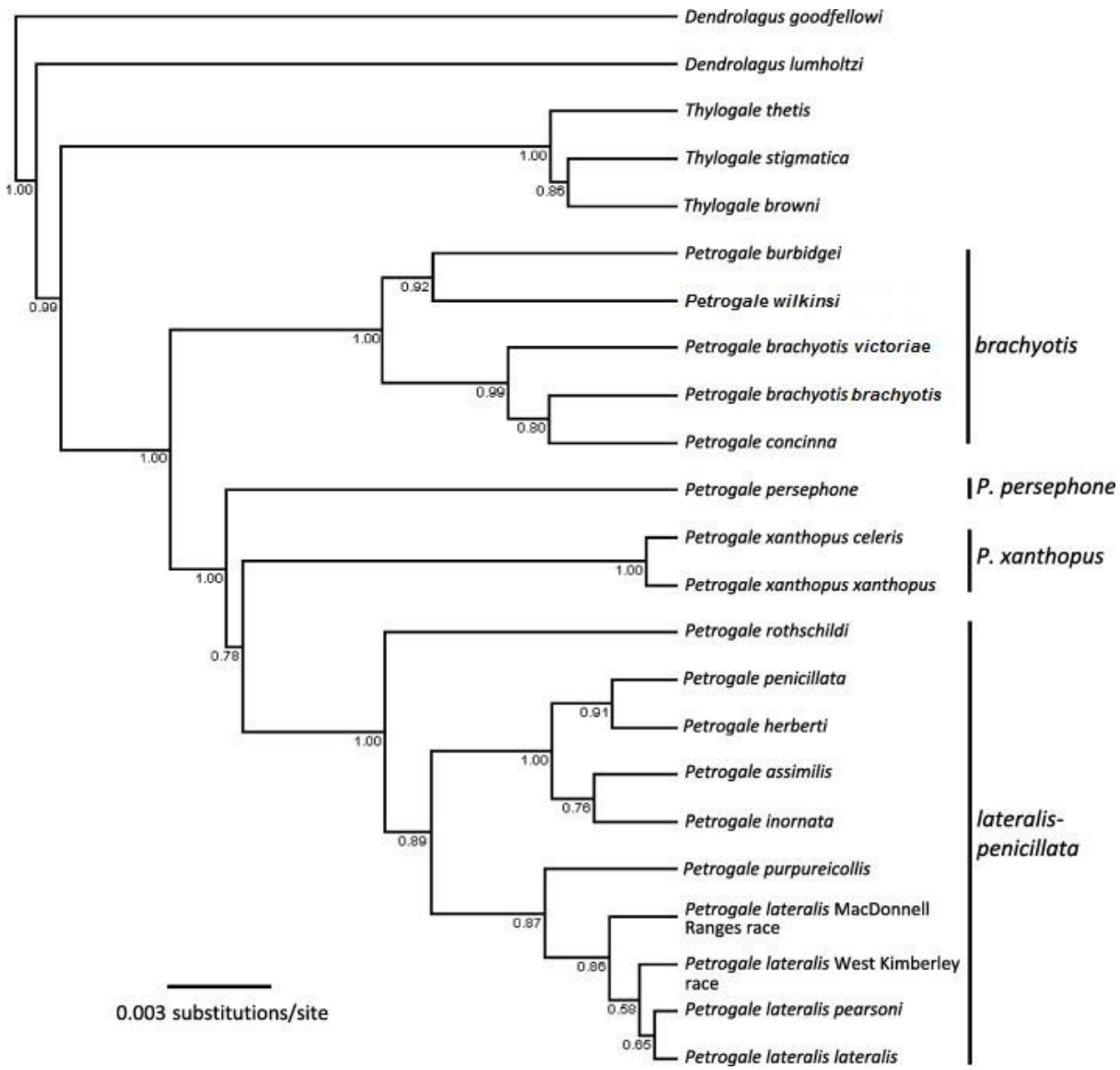
2.6.2 Conclusion

The findings of this study indicate that the parasites of *Petrogale* are highly specific and that host phylogeny and biogeography have contributed to the diversification of *Petrogale* parasite communities. With seven threatened *Petrogale* species reduced to small, isolated and fragmented populations with diminishing genetic diversity and continuing impacts of human expansion and climate change, the highly specialized parasite communities of rock-wallaby populations are also at risk. To minimise loss of biodiversity, an integrated framework that includes hosts and their associated symbionts and specialised parasite communities should form part of conservation strategies.

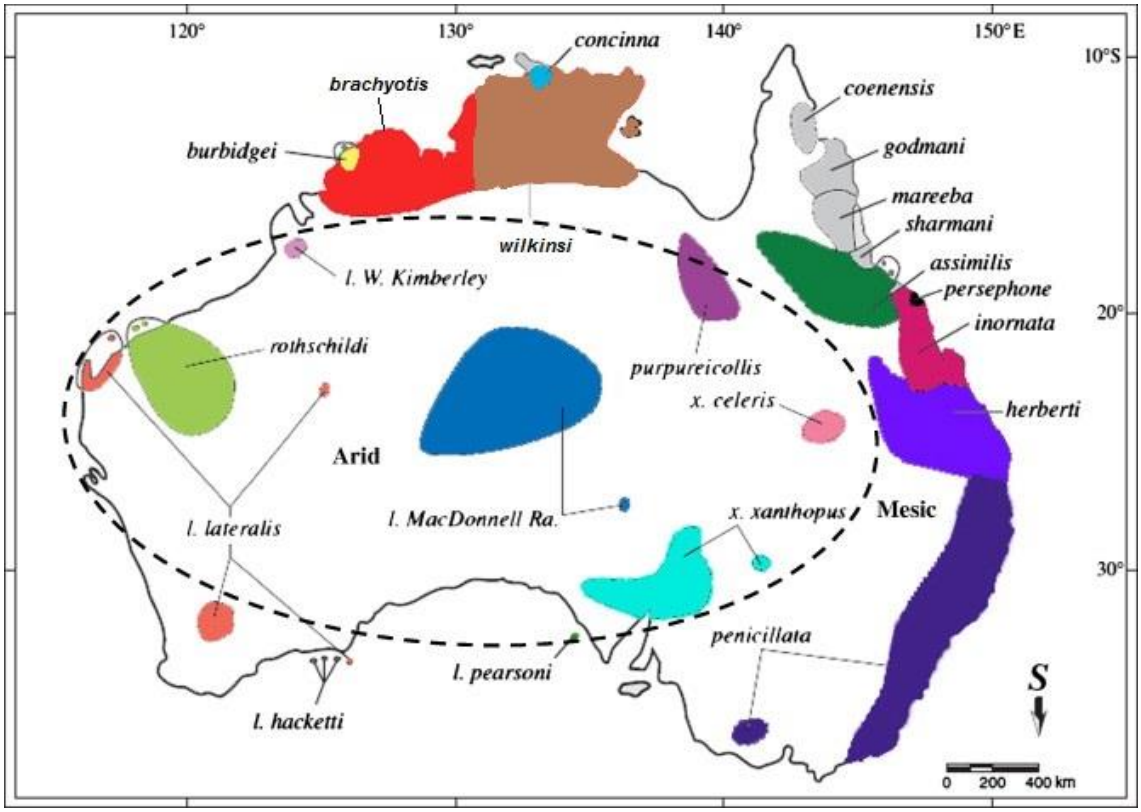
2.6.3 Acknowledgements and declaration of interest

This research was funded as part of a PhD scholarship by the Australian Research Council, Office of Environment and Heritage and the Australian Museum through the ARC Linkage program issued by Macquarie University, Project ID: LP110200569. The authors declare there is no conflict of interest.

2.7 Supplementary material



Supplementary Figure 2.1: Relationships amongst thirteen *Petrogale* species (including eight subspecies) in a BEST species tree (phylogram) using both mitochondrial and nuclear sequence data, including posterior probabilities and major clades indicated by their taxon group. *Dendrolagus* spp. are used as the outgroup. Figure modified from Potter *et al*. (2012).



Supplementary Figure 2.2: The distributions of all *Petrogale* taxa in Australia with the arid zone indicated by the dotted line, modified from Potter *et al*. (2012) and Eldridge & Close (1993) to include estimated habitat range of the newly recognised species *P. wilkinsi*, previously included under *P. brachyotis* (Potter *et al*. 2015). In the case of *P. brachyotis*, the distribution includes its subspecies.

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3 Diversity of *Cryptosporidium* in brush-tailed rock-wallabies (*Petrogale penicillata*) managed within a species recovery program

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Keywords: *Cryptosporidium*, molecular detection, brush-tailed rock-wallaby, conservation management

Published in: *International Journal for Parasitology: Parasites and Wildlife* (2015) 4 (2), 190-196.

DOI: 10.1016/j.ijppaw.2015.02.005

3.1 Abstract

Host-parasite relationships are likely to be impacted by conservation management practices, potentially increasing the susceptibility of wildlife to emerging disease. *Cryptosporidium*, a parasitic protozoan genus comprising host-adapted and host-specific species, was used as an indicator of parasite movement between populations of a threatened marsupial, the brush-tailed rock-wallaby (*Petrogale penicillata*). PCR screening of faecal samples (n = 324) from seven wallaby populations across New South Wales, identified *Cryptosporidium* in 7.1% of samples. The sampled populations were characterised as captive, supplemented and wild populations. No significant difference was found in *Cryptosporidium* detection between each of the three population categories. The positive samples, detected using 18S rRNA screening, were amplified using the actin and *gp60* loci. Multi-locus sequence analysis revealed the presence of *Cryptosporidium fayeri*, a marsupial-specific species, and *C. meleagridis*, which has a broad host range, in samples from the three population categories. *Cryptosporidium meleagridis* has not previously been reported in marsupials and hence the pathogenicity of this species to brush-tailed rock-wallabies is unknown. Based on these findings, we recommend further study into *Cryptosporidium* in animals undergoing conservation management, as well as surveying wild animals in release areas, to further understand the diversity and epidemiology of this parasite in threatened wildlife.

3.2 Introduction

Disease emergence presents a significant risk to the conservation of endangered wildlife. The risks of disease are leading to growing concern of the cost-benefit efficiency of the supplementation strategy (Kock *et al*., 2010). Species recovery actions such as the supplementation of dwindling populations with captive bred animals may introduce parasites atypical to the recovery species or exacerbate prevalence of existing pathogens due to stress and immune status of captive bred individuals, which may spread these pathogens into its new environment (Moberg, 1985, Cunningham, 1996). Control of disease risks requires a sound understanding of host-parasite interactions, both in threatened species and of hosts that may contribute to disease emergence. Further, parasites specific to the target species may not survive translocation or other conservation processes, thereby unbalancing the natural host-parasite relationship (Moir *et al*., 2012).

Cryptosporidium, a protozoan parasite with a broad vertebrate host range and variable host specificity, represents a potential indicator of disease risks associated with conservation management. This research strategy is particularly applicable to threatened Australian marsupials where the occurrence of human derived *Cryptosporidium* species has not been conclusively determined (Hill *et al*., 2008, Ng *et al*., 2011, Dowle *et al*., 2013).

Of the 27 described *Cryptosporidium* species (Ryan *et al*., 2014, 2015), twelve have been reported in both humans and other hosts: *C. parvum, C. hominis, C. ubiquitum, C. andersoni, C. bovis, C. cuniculus, C. muris, C. canis, C. felis, C. meleagridis, C. suis* and *C. fayeri* (Xiao *et al*., 2001, Gatei *et al*., 2002, Xiao, 2002, Leoni *et al*., 2006, Robinson *et al*., 2010, Waldron *et al*., 2010). Each of the *Cryptosporidium* species reported in humans have been found in the Australian environment (Ryan & Power, 2012, Abeywardena *et al*., 2013, Nolan *et al*., 2013) though human infections in Australia are predominantly *C. parvum* and *C. hominis* (Waldron *et al*., 2011).

Despite *Cryptosporidium* being identified in 16 marsupial species from 7 families (reviewed in O'Donoghue, 1995, Power, 2010), identifications of *Cryptosporidium* to species level is limited to recent studies employing molecular tools (Warren *et al*., 2003, Hill *et al*., 2008, Power & Ryan, 2008, Ryan *et al*., 2008, Yang *et al*., 2011). Following molecular identification, marsupials were found to be susceptible to two host-adapted *Cryptosporidium* species, *C. fayeri* and *C. macropodum* (Power & Ryan, 2008, Ryan *et al*., 2008). Several other host-specific genotypes have also been described in marsupials including brushtail possum genotype I from common brushtail possums (*Trichosurus vulpecula*) (Hill *et al*., 2008) and kangaroo genotype I from western grey kangaroos **(***Macropus fuliginosus*) (Yang *et al*., 2011).

Although there are reports of *C. parvum* and *C. hominis* in marsupials, these are based only upon a molecular signature from a faecal DNA sample, and an infection has never been confirmed using other methods such as parasite isolation (Hill *et al*., 2008, Ng *et al*., 2011, Dowle *et al*., 2013). The molecular detection of *C. parvum* and *C. hominis* in marsupial hosts has also been associated with an inability to confirm at greater than a single locus, namely the 18S rRNA. Passage of *C. parvum* or *C. hominis* oocysts through the marsupial gut is the likely reason for identifications of these *Cryptosporidium* species in marsupials (Dowle *et al*., 2013). The only confirmed case of *Cryptosporidium* infection in a marsupial that was not host specific was an infection of *C. muris* in captive greater bilbies (*Macrotis lagotis*) being bred for release into natural habitat (Warren *et al*., 2003).

Here we use molecular methods to detect and identify *Cryptosporidium* in the brush-tailed rock-wallaby (BTRW), *Petrogale penicillata*. This species is listed as ‘endangered’ in New South Wales, Australia (NSW *Threatened Species Conservation Act* 1995) and ‘near threatened’ on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species across eastern Australia (IUCN, 2013). There is an approved NSW

Recovery Plan for the species (DECC, 2008), as well as an approved National Recovery Plan (Menkhorst & Hynes, 2010). These plans identify supplementation of small colonies with captive bred individuals as an important recovery strategy and over the last few years several translocations of individuals between captive breeding facilities and wild populations have occurred (Menkhorst & Hynes, 2010). As rock-wallaby populations have experienced variable levels of human intervention, studying their parasites provides a platform to examine the effect of conservation management on the host-parasite relationship. Hence, our aim was to detect and identify *Cryptosporidium* species infecting wild, captive bred, and supplemented brush-tailed rock-wallaby populations.

3.3 Methods

3.3.1 Sample collection and sites

Brush-tailed rock-wallabies were once abundant in south-eastern Australia but are now reduced to fragmented populations in New South Wales and Victoria (Eldridge & Close, 2005). Dispersal between populations, which are located in steep, rocky habitats, is rare (Browning *et al*., 2001). For this study seven BTRW sites were sampled between March 2010 and July 2013 (Table 3.1). Sample collection dates were spread evenly across three seasons (autumn, summer and winter) with ~10 samples collected in Spring (2010 and 2012), spread evenly across the four years. The origin of each population varied and included three categories: one site with a BTRW population kept in a captive breeding facility (captive bred), sites where free-ranging populations had been supplemented with captive bred individuals (supplemented) and two pristine sites with only free-ranging animals (wild). Fresh faecal samples were collected in vials containing silicon beads from each site opportunistically from unknown individuals during routine colony management by Office of Environment and Heritage staff and were then stored at 4°C until further processing. The

highest number of samples was obtained from Square Top in Warrumbungle National Park since this was a major release site.

3.3.2 DNA extraction and PCR screening

Genomic DNA was extracted from faecal material (~150 mg) using the ISOLATE Fecal DNA kit (Bioline, London, UK) following manufacturer's instructions. The extracted DNA was stored at -20°C until further analysis. Directly prior to each PCR the DNA samples were treated with GeneReleaser (BioVentures, Inc., TN, USA) by combining equal volumes of DNA and GeneReleaser and subjecting the mixture to 7 min in a 500 W microwave.

3.3.3 PCR screening at the 18S rRNA locus

DNA samples were initially screened for *Cryptosporidium* using nested PCR to amplify a partial fragment of the 18S rRNA. The primary reaction followed the methodology of Xiao *et al*. (1999) but with a lower $MgCl_2$ concentration (2 mM). The secondary reaction comprised the primers 18S IF and 18S IR and followed the method of Morgan *et al*. (1997). PCRs were performed using Red Hot Taq DNA polymerase (ThermoFisher Scientific, Waltham, MA, USA) as previously described (Hill *et al*., 2008). Both reactions were modified to increase specificity for *Cryptosporidium* by lowering the concentration of dNTPs to 50 µM.

Longer 18S rRNA fragments were generated for samples testing positive for *Cryptosporidium* using the 18S IF and 18s IR primer set. The longer fragments were amplified using the primers of Xiao *et al*. (1999) for both primary and secondary reactions, following conditions as previously described by Waldron *et al*. (2011) inclusive of dNTP and $MgCl_2$ concentrations as described above.

3.3.4 PCR amplification at confirmatory loci

To confirm 18S rRNA positives, DNA samples were screened at two additional loci, actin and glycoprotein 60 (*gp60*). For the actin locus, a nested protocol (Sulaiman *et al*., 2002) was performed with minor modifications. To improve specificity for *Cryptosporidium*, the concentration of $MgCl_2$ was lowered to 2 mM, dNTPs to 50 µM and the annealing temperature raised to 54°C in the secondary reaction. All amplifications were performed using Red Hot Taq DNA Polymerase.

Amplification of the *gp60* locus was achieved using a nested protocol with primary amplification achieved using the primers outF and outR (Power *et al*., 2009) and secondary reactions using ATGF and StopR (Waldron *et al*., 2009). Red Hot Taq was used for both amplifications. All PCR reactions performed included a negative control (H_2O) and a positive control of DNA extracted from purified oocysts of *C. parvum*.

3.3.5 Sequencing of positive samples

All amplicons generated for 18S rRNA, actin and *gp60* were sequenced to enable *Cryptosporidium* species identification. Amplicons from each of the four PCRs which contained a band of the expected size when resolved by electrophoresis (2% agarose in TBE with SYBR Green staining) were purified using the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands). Purified amplicons were sequenced in both directions (Macrogen, Seoul, Korea) using their respective primers used in PCR for amplification, with the exception of the short fragment of the 18S rRNA, which were only sequenced with the primer 18S IF (Morgan *et al*., 1997).

3.3.6 Phylogenetic analysis

To enable species identification within a phylogenetic framework, samples positive for 18S rRNA (~825 bp) were trimmed to the same length and aligned with *Cryptosporidium* reference sequences from GenBank using ClustalW (Larkin et al., 2007). A phylogenetic tree was constructed based on this alignment using Neighbour-joining. Sequences generated in this study have been submitted to GenBank under accession numbers KP730299-KP730329.

3.3.7 Statistical analysis

To test differences of *Cryptosporidium* prevalence between sites and site categories, samples were tested at the 18S rRNA (~298bp) locus for presence or absence and checked for significant differences with a non-parametric test (Kruskal-Wallis) in Minitab (version 17.1.0, Minitab Inc.).

3.4 Results

3.4.1 *Cryptosporidium* screening

DNA was extracted from 324 samples and screened for *Cryptosporidium* using 18S rRNA PCR. Of the 324 screened faecal samples, 43 contained the expected amplicon. DNA sequencing and Blast searches identified 23 samples as being *Cryptosporidium,* giving a total positive identification rate of 7.1% in BTRW. Those samples that did not return as *Cryptosporidium* were identified as various *Entamoeba* sp. *Cryptosporidium* positive samples were obtained from three site types (captive bred, supplemented and wild). Positives were found to be present across most study sites except for Jenolan Caves. There was no significant difference in *Cryptosporidium* prevalence between captive bred, wild and supplemented as categories (Kruskal-Wallis non-parametric test, H = 0.380, d.f. = 2, p = 0.150). However, there was a significant difference between the sites (H = 23.5, d.f. = 6, p = 0.001). Kangaroo Valley Creek had the highest prevalence of positive samples (40%), but

this site had the lowest amount of samples tested (n = 10; Table 3.1). When KV Creek was removed from the analysis, there was no significant difference between sites (H = 7.72, d.f. = 5, p = 0.173), thus the significant difference between sites may be due to differences in sample size in this population.

Table 3.1: Frequency of *Cryptosporidium* at the different loci per screened site and site category. Samples at the loci (18S rRNA, actin and *gp60*) were deemed as positive after DNA sequencing. All sites are in New South Wales; the precise location is withheld for some sites for the safety of the animals. KV denotes 'Kangaroo Valley'.

Site	**Site Type**	**No. of samples**	**18S rRNA (298 bp)**	**18S rRNA (825 bp)**	**Actin**	***gp60***
KV Mountain	Wild	55	7	7	2	3
KV River	Supplemented	43	2	1	0	1
KV Creek	Supplemented	10	4	3	0	0
Nattai	Wild	30	3	3	1	1
Square Top	Supplemented	123	5	4	0	0
Waterfall Springs	Captive bred*	39	2	2	1	2
Jenolan Caves	Supplemented	24	0	0	0	0

* Wallabies in a captive breeding facility

3.4.2 Species identification at the 18S rRNA locus

From the initial positive samples (n = 23), 20 samples yielded sequence data for the larger 18S rRNA fragment (825 bp), which was used to generate a phylogeny (Fig. 3.1). Four samples from supplemented sites and three samples from wild sites clustered with the *C. parvum* and *C. hominis*. Three samples from supplemented sites, one from a captive bred site and three samples from wild sites grouped with the marsupial-specific species *C. fayeri* and *C. macropodum*. A further four samples from a wild site and one from a captive-bred site grouped with *C. meleagridis*.

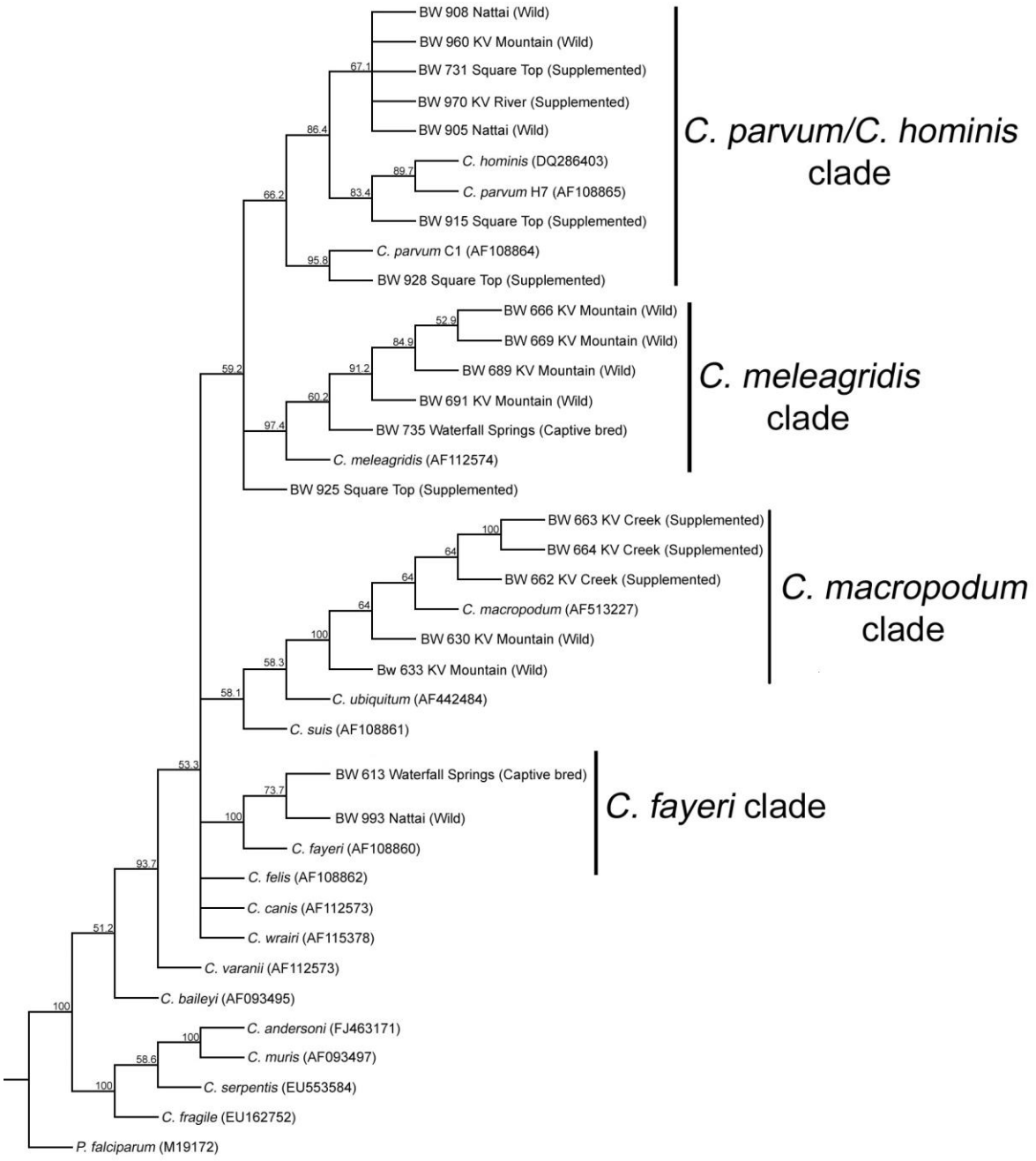

Figure 3.1: Phylogenetic tree based on neighbour-joining with bootstrap test (1,000 replicates, displayed at nodes) using the 18S rRNA locus (878 bp) of 20 BTRW *Cryptosporidium* isolates and 17 reference sequences retrieved from GenBank with accession numbers and *Plasmodium falciparum* as the outgroup. KV denotes 'Kangaroo Valley'.

3.4.3 Species confirmation using actin and *gp60*

Sequence analysis at the actin and *gp60* loci resulted in amplicons and sequence data from only eight samples across all loci (Table 3.2). At the actin locus, although 15 samples generated a band of the expected size (~1066 bp), only four were identified using BlastN searches as *Cryptosporidium,* with two samples being *C. fayeri* and two being *C. meleagridis*. For *gp60* seven samples generated an amplicon with three samples assigned to *C. fayeri* and four to *C. meleagridis* (Table 3.2). However, the four samples from Kangaroo Valley Mountain identified as *C. meleagridis* may represent the same individual sampled twice over two time points. For those samples identified at the 18S rRNA as *C. hominis* and *C. parvum* neither actin nor *gp60* could be amplified. An exception was sample BW# 973 identified as *C. hominis* at the 18S rRNA and *C. fayeri* by *gp60* sequencing.

Table 3.2: Species identification across three loci (18S rRNA, actin and *gp60*) for the samples positive at the 18S rRNA locus for *C. fayeri* and *C. meleagridis*. Samples identified at the 18S rRNA locus as other *Cryptosporidium* species (Fig. 3.1) were omitted from this table since they could not be amplified at other loci. ID% denotes the similarity of the match of the consensus sequence in GenBank. NP indicates no product was amplified. KV denotes 'Kangaroo Valley'.

BW #	**Site**	**Site Type**	**18S rRNA (298bp)**	**ID%**	**18S rRNA (825bp)**	**ID%**	**Actin**	**ID%**	***gp60***	**ID%**
613	Waterfall Springs	Captive bred	*C. fayeri*	99.6%	*C. fayeri*	99.5%	*C. fayeri*	99.9%	*C. fayeri* (subtype A10)	99.7%
666	KV Mountain	Wild	*C. meleagridis*	99.2%	*C. meleagridis*	99.9%	NP	NP	NP	NP
669	KV Mountain	Wild	*C. meleagridis*	99.8%	*C. meleagridis*	99.6%	*C. meleagridis*	99.9%	*C. meleagridis* (subtype IIIgA)	91.4%
689	KV Mountain	Wild	*C. meleagridis*	99.6%	*C. meleagridis*	99.9%	*C. meleagridis*	99.7%	*C. meleagridis* (subtype IIIgA)	91.2%
691	KV Mountain	Wild	*C. meleagridis*	99.2%	*C. meleagridis*	99.7%	NP	NP	*C. meleagridis* (subtype IIIgA)	91.6%
735	Waterfall Springs	Captive bred	*C. meleagridis*	99.1%	*C. meleagridis*	99.8%	NP	NP	*C. meleagridis* (subtype IIIbA)	88.8%
973	KV River	Supplemented	*C. hominis*	100.0%	NP	NP	NP	NP	*C. fayeri* (subtype A10)	99.9%
993	Nattai	Wild	*C. fayeri*	99.6%	*C. fayeri*	99.4%	*C. fayeri*	99.4%	*C. fayeri* (subtype A7)	99.8%

3.5 Discussion

The prevalence of *Cryptosporidium* in BTRW (7.1%) is consistent with observations of *Cryptosporidium* in other marsupials which range between 6.7% and 12.2% (Power *et al*., 2004, Hill *et al*., 2008, Yang *et al*., 2011, Dowle *et al*., 2013). Here *Cryptosporidium* prevalence in BTRW is based on sequence identifications using a 298 bp fragment of the 18S rRNA. PCR is commonly employed for detection of *Cryptosporidium* in faecal samples as this approach has greater sensitivity than microscopy, both in detection and identification of species (Fall *et al*., 2003, Power *et al*., 2003, Ryan *et al*., 2008, Dowle *et al*., 2013). In our study a larger fragment (~825 bp) failed to amplify three samples confirmed as *Cryptosporidium* using the smaller fragment, indicating that selection of optimal

amplification methods should be considered when undertaking molecular detection of this parasite.

Despite no significant difference in the detection of *Cryptosporidium* between captive bred and free ranging animals, the identity of *Cryptosporidium* species in BTRW determined by sequencing raises concern for the health status of captive and wild BTRW. *Cryptosporidium fayeri* has previously been identified in six marsupial hosts including the related yellow-footed rock-wallaby *P. xanthopus* (Morgan *et al*., 1997, Power *et al*., 2003, 2009, Ryan *et al*., 2008, Power, 2010, Yang *et al*., 2011, Nolan *et al*., 2013). *Cryptosporidium fayeri* does not appear to cause disease in marsupials (Ryan *et al*., 2008). *Cryptosporidium meleagridis* has been identified in a range of vertebrates, including avian and mammalian hosts, as well as humans (Akiyoshi *et al*., 2003, Xiao *et al*., 2004). While *C. meleagridis* is the most common infection of *Cryptosporidium* in humans after *C. parvum* and *C. hominis* (Elwin *et al*., 2012), human infections are rare in Australia (Waldron *et al*., 2011). Consequently, it is unlikely that the captive bred animals were infected from human sources, but by other host species, such as free ranging birds, inhabiting the captive breeding site. The wild site, where most of the *C. meleagridis* isolates were found, is secluded from humans and thus transmission between humans and BTRW is unlikely. The unlikeliness of this transmission route is supported by the *gp60* analysis.

The *C. meleagridis gp60* sequences from BTRW isolates displayed greater genetic similarity to *gp60* sequences from avian hosts (Stensvold *et al*., 2014), yet they were distinct from described sequences, indicating a new *gp60 C. meleagridis* subtype. This finding is the first report where a zoonotic species of *Cryptosporidium* was confirmed across multiple loci in a wild marsupial host. As such, much is unknown about the diversity and pathogenicity of *C. meleagridis* in wild marsupials and thus further study is required to understand the extent to which this species has penetrated marsupial hosts and likely transmission routes.

Cryptosporidium parvum and *C. hominis* were also identified in BTRW samples; however, these identifications were only possible at a single locus, the 18S rRNA. Only one of these samples could be amplified at one of the two confirmatory loci where it was typed as *C. fayeri*. Both *C. parvum* and *C. hominis* have been reported in a range of marsupials but similar to this study, other studies also failed to confirm identifications at loci other than the 18S rRNA (Hill *et al*., 2008, Ng *et al*., 2011, Dowle *et al*., 2013). Some isolates were inferred to be *C. ubiquitum* and *C. macropodum* through a GenBank match at the 18S rRNA locus but failed to amplify at subsequent loci (Figure 3.1). While *C. macropodum* is specific to marsupials, particularly macropods (Power & Ryan, 2008), *C. ubiquitum* is typical to cattle but is commonly identified in humans as well (Fayer *et al*., 2010). So far, no report has been made of *C. ubiquitum* in marsupials (Ryan *et al*., 2012) and thus it is more likely these samples were more closely related to *C. macropodum* from their position in the phylogenetic tree (Fig. 3.1). One sample from a supplemented site (BW# 973) matched *C. hominis* at the 18S rRNA locus and *C. fayeri* at the more specific *gp60* locus. This is likely due to the initial screening method which used a small region of the 18S rRNA locus (298 bp) which shares little genetic variation between *C. hominis* and *C. fayeri* (Ryan *et al*., 2008). Furthermore, the primers for this region are not specific to any *Cryptosporidium* species but aims to screen generally for this genus (Morgan *et al*., 1997, Xiao *et al*., 1999). Therefore, the analysis of only this particular region of the 18S rRNA cannot accurately or reliably identify the *Cryptosporidium* species, which highlights the need for both the sequencing at a larger region of the 18S rRNA to allow for greater genetic distinction, as well as the need for multi-locus genotyping.

Failure to amplify *C. parvum* and *C. hominis* isolates from marsupials at other loci has been attributed to low numbers of oocysts and the multi copy nature of the 18S rRNA locus compared to single copy confirmatory loci (Hill *et al*., 2008, Power *et al*., 2009, Ng *et al*.,

2011). Indeed, oocyst counts in possums and bandicoots confirm low oocyst numbers (Hill *et al*., 2008, Dowle *et al*., 2013). The question remains if the presence of these human infective species is merely passage of oocysts through the marsupial gut or a true infection. This process is known as pseudoparasitism, where the host ingests oocysts or eggs of a parasite that is not suited to the host and it is simply passaged through the host's digestive system (Gressler *et al*., 2009, Pinto *et al*., 2014). Pseudoparasitism needs to be taken into account when considering the identification of human specific parasites in marsupials.

Whole genome amplification could be employed to boost amplification of low oocyst numbers. This method has previously proved successful on clinical samples of *C. parvum* and *C. hominis* across three loci (Bouzid *et al*., 2010). Identification at the 18S rRNA locus alone has been found to underestimate mixed infections as this technique preferentially amplifies a predominant genotype (Reed *et al*., 2002). Mixed infections of *Cryptosporidium* were considered rare but they have only been studied so far in humans, mainly AIDS patients (Cama *et al*., 2006) and children (Xiao *et al*., 2001), and in calves (Tanriverdi *et al*., 2003). The role of mixed infections in *Cryptosporidium* pathology is still unclear. No study has so far described mixed infections in marsupials (Ryan & Power 2012). The difficulty to amplify at discriminatory loci for genotypes such as *C. parvum* in marsupials highlights the need to identify *Cryptosporidium* using a multi-locus approach.

Another difficulty encountered in this study was the potential for pseudo-replication. When working with an endangered species one encounters issues with sample collection and numbers available for stringent analyses. For instance, the Kangaroo Valley Mountain population is estimated to comprise less than 10 individuals. As we identified *C. meleagridis* in four samples from Kangaroo Valley Mountain collected over two sampling periods, it is possible that the same individual has been sampled multiple times. A possible solution to reduce bias relative to sampling would be to apply microsatellite (MSAT) analysis to identify

individuals. This method has been widely applied to many species ranging from large carnivores to small marsupials using faecal DNA, to monitor threatened populations, analyse their genetic diversity and wide-scale demographics of large populations (Spencer *et al*., 1995, Dool *et al*., 2013, Wultsch *et al*., 2014).

The findings in this study suggested that there was no direct effect of captive breeding and translocation on *Cryptosporidium* in brush-tailed rock-wallabies. In Australia, translocation policies are developed by the representative State bodies, and veterinary screening is not mandated but is increasingly employed to monitor the health of captive bred animals before release (Short, 2009). Health screening and its relation to the success of a recovery program is further complicated by a diverse number of potential pathogens and a lack of baseline data on risks that selected pathogens may pose to wildlife species. If unusual parasite species atypical to the host group are found, such as *C. meleagridis* in BTRW, consideration as to whether the animal should be used for translocation or isolated from the population would form part of the management response. The pathology of *Cryptosporidium* in wild marsupials is also currently unknown (reviewed in Ryan & Power, 2012), making such a risk assessment difficult for BTRW. The identification of *Cryptosporidium* species with varying host specificity found in both captive bred and wild brush-tailed rock-wallabies indicates that further research is required into the diversity and pathology of this parasite in Australian wildlife.

3.6 Acknowledgements

Funding for this research was provided by the Australian Research Council, Office of Environment and Heritage and the Australian Museum through the ARC Linkage program. The animal ethics approval numbers for the collections are AEC 050207/02 and AEC 080728/01. The authors would like to thank Todd Soderquist, Celia Thomson, Melinda Norton and Juliet Dingle for collecting the BTRW faecal samples.

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4 Investigation into potential transmission sources of *Giardia duodenalis* in a threatened marsupial (*Petrogale penicillata*)

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Keywords: *Giardia duodenalis*, brush-tailed rock-wallaby, zoonotic transmission, molecular detection

Published in: *Infection, Genetics and Evolution* (2015) 33, 277–280.

DOI: 10.1016/j.meegid.2015.05.015

4.1 Abstract

Assemblages of the protozoan parasite *Giardia duodenalis* common in humans and domestic species are increasingly identified in wildlife species, raising concern about the spill-over of pathogens from humans and domestic animals into wildlife. Here, the identity and prevalence of *G. duodenalis* in populations of a threatened marsupial, the brush-tailed rock-wallaby (*Petrogale penicillata*), was investigated. Identification of *G. duodenalis* isolates, across three loci (18S rRNA, β-giardin and *gdh*), from rock-wallaby faecal samples (n = 318) identified an overall detection rate of 6.3%. No significant difference in *G. duodenalis* detection was found among captive, wild and supplemented populations. Isolates were assigned to the zoonotic assemblages A and B at 18S rRNA, with sub-assemblages AI and BIV identified at the β-giardin and *gdh* loci, respectively. Assemblages AI and BIV have previously been identified in human clinical cases, but also in domestic animals and wildlife. The identifications of the *Giardia* assemblages common to humans and domestic animals indicates the transmission potential of *G. duodenalis* via humans, domestic animals and other Australian wildlife.

4.2 Introduction

Giardia duodenalis (*syn. G. intestinalis* and *G. lamblia*) is a protozoan parasite that infects a broad range of mammalian hosts, including humans (Cacciò & Ryan, 2008). *Giardia* taxonomy relies heavily on molecular data which has shown *G. duodenalis* to be a species complex that is divided into assemblages (A-H) and sub-assemblages (e.g. AI to AIV) that vary in host specificity (Feng & Xiao, 2011) and pathogenicity (Read *et al*., 2002). The zoonotic assemblages A and B typically infect humans but have been found in a range of other mammals, including wildlife, while the other assemblages (C-H) are usually host-specific (reviewed in Feng & Xiao, 2011).

Spill-over of zoonotic assemblages of *G. duodenalis* into wildlife populations have been associated with agriculture and recreational activities (reviewed in Thompson, 2013). Recent molecular studies of *G. duodenalis* in terrestrial and marine wildlife have identified zoonotic assemblages (A and B) more commonly than host-specific assemblages (C-H) (Ash *et al*., 2010, Johnston *et al*., 2010, Thompson *et al*., 2010, Delport *et al*., 2014). The proximity of wild host populations to areas inhabited by humans was associated with observed increases in the prevalence of zoonotic assemblages of *G. duodenalis* (Delport *et al*., 2014).

The spill-over of zoonotic *G. duodenalis* assemblages has also occurred in Australia, where wild animals have mostly been reported to be infected with zoonotic assemblages rather than host-specific ones (McCarthy *et al*., 2008, Thompson *et al*., 2008, 2010, Ng *et al*., 2011). To date *G. duodenalis* has been reported in 27 marsupial species across 8 families (Marino *et al*., 1992, Bettiol *et al*., 1997, Buckley *et al*., 1997, Millstein & Goldsmith, 1997, Thompson *et al*., 2008, 2010, Ng *et al*., 2011), but identifications to assemblage and sub-assemblage are restricted to recent molecular studies (Adams *et al*., 2004, McCarthy *et al*., 2008, Thompson *et al*., 2008, 2010, Ng *et al*., 2011). The most frequent identifications of *Giardia* in

marsupials are of the assemblages commonly found in humans (A and B) (McCarthy *et al*., 2008, Thompson *et al*., 2008, 2010, Ng *et al*., 2011) or assemblages specific to domestic or feral animals, for example C, D (canines) and E (pigs) (Thompson *et al*., 2010, Ng *et al*., 2011). A possibly unique marsupial assemblage, the 'quenda genotype', was identified in the southern brown bandicoot (*Isoodon obesulus*), but appears to be only found in this host (Adams *et al*., 2004, Thompson *et al*., 2010). These identifications suggest there is a transmission route of *G. duodenalis* from humans and / or domesticated animals to marsupial species, as well as other wildlife, with which the marsupials can be in contact.

Management practices such as captive breeding may involve close contact between wildlife and humans, as well as other domestic and commensal animals, and thus allow for a potential transmission route for parasites (Warren *et al*., 2003). To investigate transmission routes of *G. duodenalis* in Australian wildlife, we used molecular tools to detect and characterise *G. duodenalis* isolates in a threatened marsupial species undergoing conservation management. Moreover, we investigated whether the prevalence and identity of *G. duodenalis* varied with increased exposure to humans and other animals. The brush-tailed rock-wallaby (BTRW, *Petrogale penicillata*) was chosen for this study, as it is listed as 'near threatened' (IUCN, 2013) and a state-wide Recovery Plan has been operating in New South Wales since 2005 (DECC, 2008). As part of this Recovery Program, BTRW have been bred in captivity and translocated to various locations (DEC, 2005, DECC, 2008, Menkhorst & Hynes, 2010), resulting in BTRW populations with varying levels of exposure to humans and other animals.

4.3 Methods

4.3.1 Sample collection and DNA preparation

Brush-tailed rock-wallabies (BTRW) were once abundant across south-eastern Australia but since European settlement have been reduced to fragmented populations (Eldridge & Close,

2005). Contact between these remnant populations is now rare (Browning *et al*., 2001). For this study, seven sites in New South Wales were selected and assigned to one of three categories according to its management history: a site which housed captive bred animals (captive bred), a site which comprised free-ranging animals with no direct human contact (wild) and sites where free-ranging populations had been supplemented with captive bred animals (supplemented) (Table 4.1). For each site faecal samples were opportunistically collected between March 2010 and July 2013 during routine colony management by Office of Environment and Heritage staff members who are experienced at recognising BTRW scats. Faecal samples were subsequently stored at 4°C until DNA extraction. Genomic DNA was extracted from faeces (~150 mg) using the ISOLATE Fecal DNA kit (Bioline, London, UK) following the manufacturer's instructions. The extracted DNA was subsequently stored under -20°C.

Table 4.1: *Giardia duodenalis* screening and frequency across three loci (18S rRNA, β-giardin and *gdh*) in seven sampled brush-tailed rock-wallaby populations. KV denotes 'Kangaroo Valley'.

Site	**Site Type**	**Total screened**	**18S rRNA**	**β giardin**	***gdh***
KV Mountain	Wild	55	17	0	10
KV River	Supplemented	51	13	0	5
KV Creek	Supplemented	10	2	0	1
Nattai	Wild	30	4	1	1
Square Top	Supplemented	109	24	3	15
Waterfall Springs	Captive bred	39	3	0	1
Jenolan Caves	Supplemented	24	7	0	1

4.3.2 Parasite detection and identification

DNA samples were screened at the 18S rRNA locus using a previously described nested PCR protocol (Hopkins *et al*., 1997, Read *et al*., 2002). DNA extracted from trophozoites of *G.*

duodenalis assemblage B was used as a positive control for all PCRs. Samples deemed positive at the 18S rRNA locus through gel electrophoresis (2% agarose in TBE with SYBR safe staining (Promega, Australia) were amplified at two additional loci (β-giardin and *gdh*) to identify *Giardia* assemblages. Amplification of the β-giardin (primers: B7/B759 and BG F/BG R) and *gdh* (primers: GDHeF/GDHiR and GDHiF/GDHiR) loci was achieved using nested and semi-nested PCRs respectively (Cacciò *et al*., 2002, Read *et al*., 2004, Lalle *et al*., 2005). All PCRs included a negative control (PCR-grade water) and a positive control (DNA extracted from cultured *G. duodenalis* trophozoites source). Samples displaying a single band of the expected size for each locus in gel electrophoresis were then purified using the QIAquick PCR purification kit (Qiagen, Venlo, Netherlands) and sequenced in the forward and reverse direction (Macrogen, Seoul, Korea).

The sense and anti-sense sequences were aligned manually and checked for reading errors. To identify the assemblage and sub-assemblage of the *G. duodenalis* isolates, a consensus sequence was compared to previously published *G. duodenalis* sequences in GenBank through BlastN using Geneious (version 6.1.7, Biomatters LtD, New Zealand). Sequences generated for 18S rRNA were submitted to the European Nucleotide Archive under accession numbers LN811452-LN811463 and sequences at the β-giardin and *gdh* loci were submitted to GenBank under accession numbers KP756604-KP756614.

Statistical analysis was performed in Minitab version 17.1.0 (LEAD Technologies, Inc., PA, USA). Prevalence of *G. duodenalis* detection and identification compared between sites and site categories was tested with a non-parametric Kruskal-Wallis test.

4.4 Results

4.4.1 Detection of *Giardia duodenalis*

In this study, 318 wallaby faecal samples were screened for *G. duodenalis* using a small fragment of the 18S rRNA. A positive amplicon of the correct size was detected in 70 DNA samples (22%) (Table 4.1) representing all site categories (wild, supplemented and captive bred). Based on initial prevalence estimates from PCR detection at the small 18S rRNA fragment, there was no significant difference in prevalence of *G. duodenalis* between site categories (Kruskal-Wallis non-parametric test, H = 3.90, d.f. = 2, $p = 0.142$) or between sites (H = 9.58, d.f. = 6, $p = 0.144$).

4.4.2 Identification of *Giardia duodenalis* assemblages

Sequencing of 12 of the 70 18S rRNA positives confirmed samples to be *G. duodenalis* assemblages A or B (Table 4.2). The 12 samples identified at the 18S rRNA locus were obtained from wallaby faeces from three supplemented sites (Kangaroo Valley Creek and River, and Square Top) and a captive bred site (Waterfall Springs). To gather further genetic data on the isolates, the 70 positive samples detected at the 18S rRNA locus were amplified at the β-giardin and the *gdh* loci. For β-giardin, 5 amplicons were positive with sequence analysis identifying 4 amplicons as assemblage AI. One sequenced sample was obtained from a wild site (Nattai) and three from a supplemented site (Square Top). At the *gdh* locus, 34 samples were positive and only 7 were successfully sequenced, with each sample identified as assemblage BIV. Four of these samples were from a wild site (Kangaroo Valley Mountain) and three were from supplemented sites (Kangaroo Valley River and Square Top). Thus, a total of 20 *G. duodenalis* isolates were identified across the three loci (Table 4.2).

Table 4.2: Identification of *G. duodenalis* assemblages and sub-assemblages in seven brush-tailed rock-wallaby populations, after sequencing three loci (18S rRNA, β-giardin and *gdh*). NS indicates no sequence was obtained. ID% indicates percentage of similarity to matched sequence in GenBank. KV denotes 'Kangaroo Valley'. The number in brackets denotes the GenBank accession number that matches the isolate sequence.

BW#	Site	Population Category	18S rRNA assemblage	ID%	β-giardin sub- assemblage	ID%	*gdh* sub-assemblage	ID%
631	KV Mountain	Wild	NS	NS	NS	NS	BIV (EF507672)	100.0%
667	KV Mountain	Wild	NS	NS	NS	NS	BIV (EF507672)	100.0%
672	KV Mountain	Wild	NS	NS	NS	NS	BIV (EF507672)	100.0%
675	KV Creek	Supplemented	B (JX972180)	99.4%	NS	NS	NS	NS
676	KV Creek	Supplemented	B (JX972180)	98.8%	NS	NS	NS	NS
677	KV River	Supplemented	B (JX972180)	99.7%	NS	NS	NS	NS
678	KV River	Supplemented	B (JX972180)	99.7%	NS	NS	NS	NS
679	KV River	Supplemented	B (JX972180)	99.1%	NS	NS	NS	NS
680	KV River	Supplemented	A (KJ027407)	94.9%	NS	NS	BIV (EF507665)	99.9%
683	KV River	Supplemented	A (AY775190)	97.1%	NS	NS	NS	NS
694	KV Mountain	Wild	NS	NS	NS	NS	BIV (EF507672)	100.0%
733	Waterfall Springs	Captive bred	A (KJ027407)	98.9%	NS	NS	NS	NS
734	Waterfall Springs	Captive bred	A (KJ027407)	99.6%	NS	NS	NS	NS
781	Square Top	Supplemented	A (JQ781662)	97.1%	NS	NS	NS	NS
918	Square Top	Supplemented	A (KJ027407)	99.4%	NS	NS	NS	NS
920	Square Top	Supplemented	A (KJ027407)	99.6%	NS	NS	NS	NS
922	Square Top	Supplemented	NS	NS	AI (KJ363393)	99.8%	BIV (EF507672)	100.0%
932	Square Top	Supplemented	NS	NS	AI (KJ363393)	100.0%	NS	NS
933	Square Top	Supplemented	NS	NS	AI (KJ363393)	100.0%	BIV (EF507672)	99.9%
991	Nattai	Wild	NS	NS	AI (KJ188032)	100.0%	NS	NS

Sequences generated across different loci for the same sample, resulted in an inconsistency in assigned assemblages. Three samples identified as assemblage A at the 18S rRNA and β-giardin loci were assigned to assemblage B at the *gdh* locus.

4.5 Discussion

The occurrence of *Giardia duodenalis* in BTRW faeces was 22% using 18S rRNA PCR for detection. When the identity of *G. duodenalis* was confirmed through sequencing across three

loci, the overall prevalence was 6.3% (20/318). Previous studies employing different techniques (PCR, ELISA and microscopy) found a large range of *G. duodenalis* prevalence among various marsupial hosts, ranging from 0.11% to 62% (Bettiol *et al*., 1997, Adams *et al*., 2004, McCarthy *et al*., 2008, Thompson *et al*., 2008, 2010, Ng *et al*., 2011, Ahmed *et al*., 2012). Prevalence does, however, vary with the technique used for parasite detection, with PCR found to be more sensitive than microscopy for detection of *G. duodenalis* in animals, including in marsupials (Ryan *et al*., 2005, Thompson *et al*., 2008). As shedding of *Giardia* cysts is often low (Cacciò & Ryan, 2008), high test sensitivity is essential for detecting *G. duodenalis* and thus PCR was selected for this study.

Subsequent screening of the *G. duodenalis* isolates from BTRW at each site category identified assemblages commonly found in humans (A and B). Identification at the sub-assemblage level at the β-giardin locus determined the isolates to be AI, a sub-assemblage frequently identified in non-human animals (Cacciò & Ryan, 2008), yet also described in human cases in Australia (Read *et al*., 2004). At the *gdh* locus, the isolates were assigned to assemblage BIV. However, care must be taken with assignment to assemblage B sub-assemblages as these have only been confirmed through allozyme electrophoretic studies, and assignment using allozyme electrophoresis and genotyping has not yet shown to be consistent (Feng & Xiao 2011) and thus this particular GenBank match may not be highly reliable. Assemblage B is largely restricted to humans but has been described in various kangaroos and wallabies, with assemblage B less frequently identified than assemblage A at the 18S rRNA locus (Thompson *et al*., 2008, Ng *et al*., 2011). Both assemblages AI and BIV have been described in human clinical cases in New South Wales, with BIV the most commonly identified assemblage (75%) and AI the least frequent (1%) (Asher *et al*., 2012). However, there is potential that detection of human-specific assemblages may be due to pseudoparasitism (Gressler *et al*., 2009, Pinto *et al*., 2014) and therefore,the identification of

these anthropozoonotic assemblages in marsupials needs to be considered carefully or suported with other loci or direct paraiste identification. Further study may expand upon molecular identification by trying to purify the *G. duodenalis* parasite from the faecal samples and look for the presence and infection intensity of trophozoites through microscopy, as this is the stage in the lifecycle of *G. duodenalis* that emerges from the infective cyst and parasitises the host gut (Marquadt *et al*., 2000). Therefore, the presence of trophozoites in faeces may present a stronger case for a true infection.

The presence of these anthropozoonotic *Giardia* assemblages in BTRW faeces suggests there is a spill-over of parasites from human and other animals into the environment of BTRW populations. This transmission has been detected in BTRWs from captive bred, supplemented and wild sites with no difference found in prevalence and identification between these populations in this study. This finding suggests that while active management causes no major increase in transmission of *G. duodenalis* in BTRW, there is a spill-over of *G. duodenalis* from humans and / or domestic animals to wildlife, possibly through agricultural practices or recreational activities (Thompson, 2013).

Characterisation at different loci showed an inconsistency in the identification of assemblages A and B, which has been previously described in samples from captive macropods, where assemblage A was identified at 18S rRNA and assemblages BIII and BIV at *gdh* (Thompson *et al*., 2008). Inconsistent identification of *G. duodenalis* assemblages at different loci is a common problem in diverse hosts (Traub *et al*., 2004, Cacciò *et al*., 2008, Beck *et al*., 2011). One possible explanation for this problem is a mixed infection, which has commonly been found in *G. duodenalis* (reviewed in Cacciò & Ryan, 2008). The β-giardin locus has been found to underestimate mixed infections when compared to using assemblage-specific primers at the triose-phosphate isomerase locus (*tpi*) (Geurden *et al*., 2008). The 18S rRNA region may also not effectively discriminate between assemblages due to the high level of

conserved base pairs, particularly in the short fragment typically used for *Giardia* detection (Read *et al*., 2002, Thompson *et al*., 2008). This issue highlights the need to amplify across multiple loci for *G. duodenalis*.

The identification of the zoonotic assemblages A and B of *G. duodenalis* in BTRW raises concern about the spill-over of potential pathogens from humans and domesticated animals to wildlife. As Australian wildlife is increasingly threatened by habitat fragmentation, invasive species and human activities (Department of the Environment, 2009), such spill-over events may become more frequent. Hence, understanding disease risks, gathering baseline data and establishing indicators of disease transmission is crucial for conserving Australia's threatened wildlife.

4.6 Acknowledgements

This research was funded by the Australian Research Council, Office of Environment and Heritage and the Australian Museum through the ARC Linkage program; Project ID: LP110200569. The animal ethics approval numbers are AEC 050207/02 and AEC 080728/01. The authors would like to thank Todd Soderquist, Celia Thomson, Melinda Norton and Juliet Dingle from Office of Environment and Heritage for collecting the BTRW faecal samples.

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5 Evaluation of next generation sequencing for the analysis of *Eimeria* communities in wildlife

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Keywords: next generation sequencing, microbial eukaryotes, *Eimeria,* community analysis, data analysis, wildlife

Published in: *Journal of Microbiological Methods*, 124, 1-9

DOI: 10.1016/j.mimet.2016.02.018

5.1 Abstract

Next-generation sequencing (NGS) techniques are well-established for studying bacterial communities but not yet for microbial eukaryotes. Parasite communities remain poorly studied, due in part to the lack of reliable and accessible molecular methods to analyse eukaryotic communities. We aimed to develop and evaluate a method to analyse communities of the protozoan parasite *Eimeria* from populations of the Australian marsupial *Petrogale penicillata* (brush-tailed rock-wallaby) using NGS. An oocyst purification method for small sample sizes and polymerase chain reaction (PCR) protocol for the 18S rRNA locus targeting *Eimeria* was developed and optimised prior to sequencing on the Illumina MiSeq platform. A data analysis approach was developed by modifying methods from bacterial genetic community analysis and utilising existing *Eimeria* sequences in GenBank. Operational taxonomic unit (OTU) assignment at a high similarity threshold (97%) was more accurate at assigning *Eimeria* contigs into *Eimeria* OTUs but at a lower threshold (95%) there was greater resolution between OTU consensus sequences. The assessment of two amplification PCR methods prior to Illumina MiSeq, single and nested PCR, determined that single PCR was more sensitive to *Eimeria* as more *Eimeria* OTUs were detected in single amplicons. We have developed a simple and cost-effective approach to a data analysis pipeline for community analysis of eukaryotic organisms using *Eimeria* communities as a model. The pipeline provides a basis for evaluation using other eukaryotic organisms and potential for diverse community analysis studies.

5.2 Introduction

Next-generation sequencing (NGS) platforms allow for fast, cost-effective and high-throughput genetic analyses with high sensitivity (van Dijk, 2014). In comparison, conventional (Sanger) sequencing (Sanger *et al*. 1977), including cloning and sequencing of rDNA amplicons, is slower and more expensive on a large scale and less sensitive. Due to its speed and cost-effectiveness, NGS has enabled large-scale genetic analyses such as metagenomics of bacterial communities from diverse sample sources (reviewed in Faust & Raes, 2012). However, while pipelines for analysing community data from NGS have been well established for bacteria (Cole *et al*., 2009, Schloss *et al*., 2009, Caporaso *et al*., 2010, Giongo *et al*., 2010), techniques for studying the genetic community structure of microbial eukaryotes are less established (Bik *et al*., 2012).

The few studies of protozoa using NGS have focused mainly on whole genome assembly (Guo *et al*., 2015, Hanevik *et al*., 2015). Some studies have applied the technique to examine intra-specific diversity of two important human parasites *Plasmodium* (Bunnik *et al*., 2013, Carlton *et al*., 2013, Hester *et al*., 2013, Bordbar *et al*., 2014) and *Cryptosporidium* (Grinberg *et al*., 2013, Paparini *et al*., 2015). Where studies of eukaryotes using NGS have been community focused, these have targeted free-living marine or fresh-water protozoa (Bråte *et al*., 2010, Lecroq *et al*., 2011), or metazoans such as fungi (Schmidt *et al*., 2013, Bálint *et al*., 2014) and parasitic helminths (Lott *et al*., 2015). To date, there is no well-established pipeline for analysing the community structure of microbial eukaryotic parasites.

The genus *Eimeria,* an apicomplexan protozoan parasite, was chosen as a model parasite to develop and assess a NGS analysis protocol for examining protozoan parasite communities. *Eimeria* is highly diverse with estimates of the genus comprising over a thousand species (Blake, 2015). *Eimeria* species are traditionally identified using morphology of the oocyst

(Duszynski & Wilber, 1997), which contains sporozoites (the infective stage of the parasite) (Chapman *et al*., 2013). Descriptions of *Eimeria* species also draw on information such as host specificity and geographical distribution (Duszynski & Wilber, 1997). Describing and identifying *Eimeria* species using traditional approaches can be problematic. Hosts can be infected with multiple species and the oocysts of a single species often show variation in morphological characteristics used for differentiation, while some species share highly similar morphological characteristics (Joyner, 1982, Zhao *et al*., 2001, Jeanes *et al*., 2013). Using hosts to aid identification is also complicated as *Eimeria* species can sometimes have a broad host range but usually in evolutionary related host species (Barker *et al*., 1989) and genera (Zhao *et al*., 2001, Kvičerová & Hypša, 2013). Thus, recent efforts have been made to combine morphological analysis with molecular techniques to characterise *Eimeria* species (Barta *et al*., 1991, Zhao *et al*., 2001, Power *et al*., 2009, Hill *et al*., 2012, El-Sherry *et al*., 2013, Afonso *et al*., 2014, Heitlinger *et al*., 2014).

Initial molecular studies of *Eimeria* with conventional sequencing focused on commercially important species such as pathogenic species in poultry (Chapman & Shirley, 2003, Novaes *et al*., 2012) and cattle (reviewed in Daugschies & Najdrowski, 2005), and then expanded into genetic diversity of species of non-domestic hosts (Zhao *et al*., 2001, Power *et al*., 2009, Hill *et al*., 2012, Afonso *et al*., 2014). Molecular studies of *Eimeria* utilising NGS have focused solely on commercially important species for whole genome assembly (Heitlinger *et al*., 2014, Reid *et al*., 2014) or for high-throughput transcriptomic analyses (Matsubayashi *et al*., 2013, Hong *et al*., 2014). There has been no study to date involving NGS for a community analysis of *Eimeria* species.

With *Eimeria* as a model organism, we aimed to develop a methodology to analyse the genetic structure of communities of microbial eukaryotes using NGS. To evaluate the effectiveness of PCR amplification, sequencing platform and data analysis protocols, we

investigated *Eimeria* communities from populations of brush-tailed rock-wallabies (*Petrogale penicillata*), a marsupial host in which multiple species of *Eimeria* have been described (Barker *et al*., 1988).

5.3 Methods

5.3.1 Sample collection, oocyst purification and DNA extraction

Faecal samples of brush-tailed rock-wallabies (BTRW) were collected opportunistically over a period of five years between March 2009 and December 2014 during routine site management by Office of Environment and Heritage staff at nine sites in New South Wales, Australia (Table 5.1). Faecal samples were stored in plastic vials with silicon beads for desiccation to prevent growth of fungi and other potential PCR inhibitors, then transported to the lab and stored at 4°C.

Eimeria oocysts were purified from faecal samples using a sucrose flotation technique (Truong & Ferrari, 2006), modified for a smaller faecal volume (0.5 g). Faecal material was sieved (100 µm) and centrifuged (2500 g, 10 min), and the resulting pellet mixed with 5 ml sterilised sucrose solution (55% w/v) (Sigma-Aldrich, MO, USA). The faecal-sucrose mix was overlaid with 5 ml sterile water and centrifuged (2500 g, 30 min). Oocysts were aspirated from the middle layer, resuspended in 15 ml of water and centrifuged (2500 g, 10 min). The resulting pellet was suspended in 200 µl distilled water (MilliQ water, autoclaved), the presence of oocysts confirmed and number of oocysts per gram counted through bright-field microscopy (Olympus Corporation, Tokyo, Japan).

To extract DNA from purified oocysts, 50 µL of the oocyst suspensions were added to a 0.5 ml tube containing ~100 mg of glass beads (425-600 µm, Sigma-Aldrich, MO, USA). The tubes were vortexed for 1 min to break the oocyst wall (Ogedengbe *et al*., 2011). DNA

was extracted using the prepGEM kit according to the manufacturer's instructions (ZyGEM Corporation, Hamilton, New Zealand) and kept at -20°C (Power *et al*., 2009).

5.3.2 PCR optimisation for Illumina MiSeq

Primers targeting a region of the 18S rRNA were designed using aligned *Eimeria* species sequences from GenBank (Supplementary Table 5.1) in Primer3 (Geneious, Biomatters Ltd., New Zealand) with a target fragment size of ~500 bp and a primer melting temperature of 60-65°C. The region selected includes the hypervariable V4 region of the 18S rRNA locus (Gagnon *et al*., 1996), which provides a good estimate of phylotype richness within apicomplexans (Morrison *et al*., 2004). The overhang adapters compatible with Illumina MiSeq index and sequencing adapters were added at the 5' end for both the forward 365F (5'-GAGCCTGAGAAACGGCTACC-3') and reverse primer 872R (5'-TACGAATGCCCCCAACTGTC-3') according to instructions by the Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Sydney, Australia. PCR conditions were optimised using a positive control of confirmed *Eimeria trichosuri* DNA extracted using the above protocol from common brushtail possum (*Trichosurus vulpecula*) faecal samples. The PCR protocol was optimised using two different primer concentrations (0.2 and 0.4 µM), a gradient of Mg^{2+} concentrations (2 to 4 mM with 1 mM increments), number of cycles (30 to 50 with 2 cycle increments) and the annealing temperature (54 to 64°C with 2°C increments). Final conditions for amplifications were comprised of 2.5 µl template DNA, 0.4 µM of each primer, 2 mM of $MgSO_4$, 0.2 mM of each dNTP, and 1 U of Platinum Taq DNA Polymerase HiFi (Life Technologies, Mulgrave, VIC, Australia) and 1X High Fidelity PCR buffer, made up with PCR-grade water to 25 µl per reaction. After an initial denaturation of 94°C for 2 min, the program was comprised of 45 cycles of 94°C for 15 sec, 58°C for 30 sec and 68°C for 1 min, followed by a final extension of 68°C for 7 min. A subset of samples was subsequently selected for a nested PCR protocol, with the primary reaction following a

previously described protocol (Zhao and Duszynski, 2001), modified to 30 cycles with the extension temperature adjusted to 68°C and optimised Mg^{2+} concentration (2 mM). The secondary reaction followed the same program as the single PCR reaction, except it consisted of 35 cycles instead of 45.

Amplicons were resolved using 2% agarose gel electrophoresis and samples purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). DNA concentration was measured and standardised to 5-20 ng/µl using a Qubit (Life Technologies, Mulgrave, VIC, Australia) through dilution with PCR-grade water. Genomic libraries were prepared with the Nextera XT Index Kit (Illumina, San Diego, USA). Sequencing was performed in 300 bp paired-ends reads with an Illumina MiSeq sequencing platform by the Ramaciotti Centre for Gene Function Analysis.

5.3.3 Sequence analysis

The methodology is described in Figure 5.1. Data were filtered following the recommendations for NGS data clean-up for eukaryotes (Nilsson *et al*., 2011, Lott *et al*., 2015), adapted for the *Eimeria* 18S rRNA locus. Using SeqMan NGen (DNASTAR, Madison, USA), adapter sequences and terminal nucleotides were trimmed (30 bp from the 5' and 3' end) and low quality bases were removed (minimum quality score = 30) from the reads. Contigs were created using *de novo* assembly of paired-end reads with a minimum match percentage of 99% and with removal of small reads (<100 bp) and low frequency reads (< 100 sequences). The contigs of each sample were imported into Geneious and aligned against a reference sequence of *E. trichosuri* (accession # FJ829320) for correct orientation. Sequences were imported into MOTHUR and further de-noised with pre-clustering, unique sequence generating algorithms and removal of chimeric sequences using the Chimera.bellerophon function (Schloss *et al*., 2009). The MOTHUR code is provided in

Supplementary Material (section 5.7). Sequences were then assigned to operational taxonomic units (OTU) through calculation of pairwise sequence distances without penalisation for end gaps and the OTUs were clustered at two different minimum identity thresholds, 97% and 95%, with the furthest neighbour algorithm. A consensus sequence was created in Geneious for each OTU from both similarity thresholds and matched to similar sequences in GenBank (NCBI) using the BlastN function. All sequences generated in this study were deposited in the NCBI sequence read archive under the study accession number SRP062125.

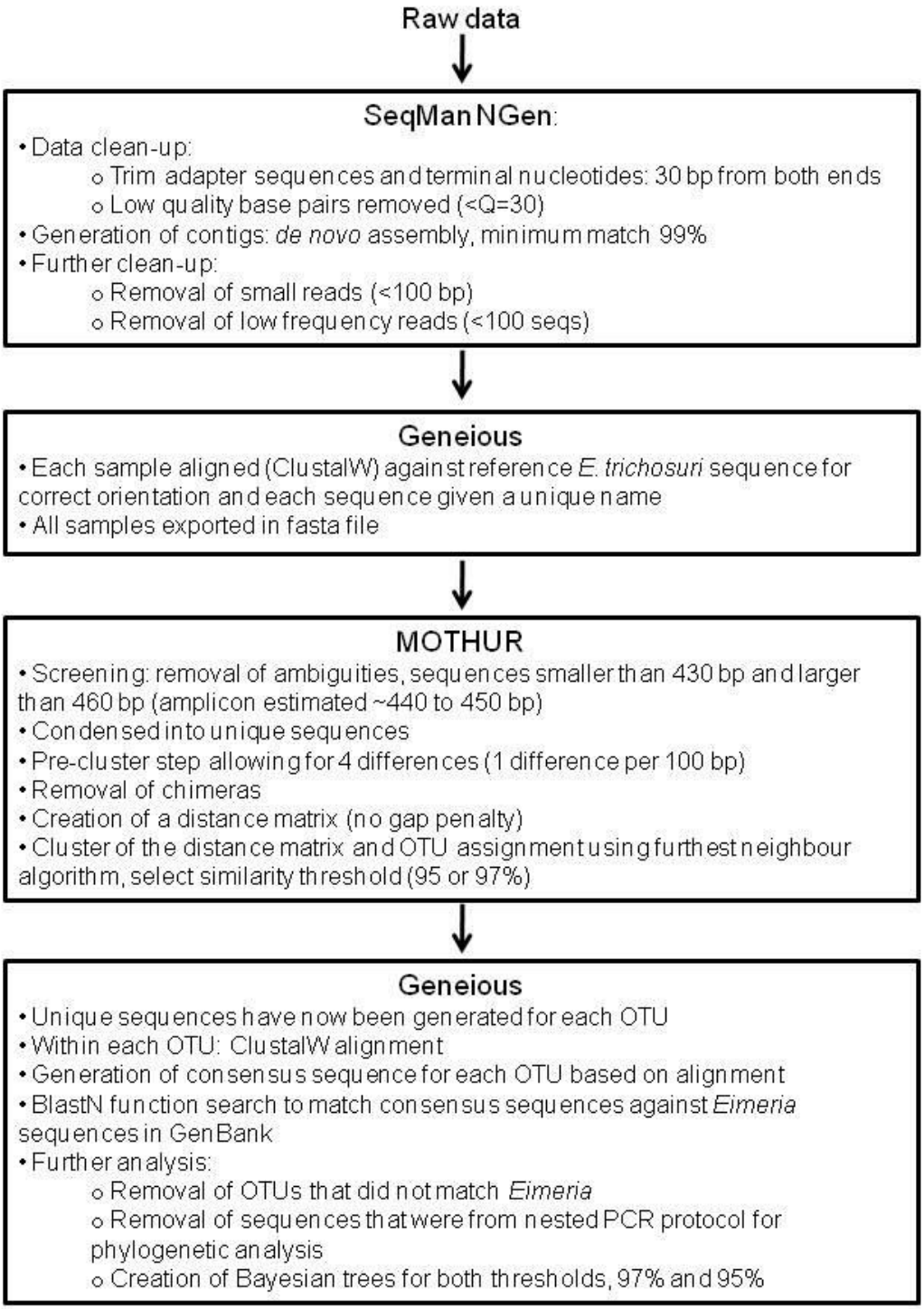

Figure 5.1: Flowchart of the methodology of data analysis post-Illumina MiSeq sequencing, including the programs used in the process.

5.3.4 Phylogenetic analysis

The phylogenetic relationship of the OTUs and *Eimeria* reference sequences from GenBank were analysed using a Bayesian Markov Chain Monte Carlo (MCMC) analysis. *Eimeria* reference sequences were selected to primarily represent mammalian-specific species (33 sequences), with six poultry-specific species also included. A general time reversible (GTR) model was selected for this analysis, with a gamma distribution with four rate categories, based on evaluation through JModelTest 2.1.8 using the Bayesian information Criterion (BIC) (Darriba *et al*. 2012). The Bayesian tree was created in Geneious using Mr. Bayes 3.2.2 (Huelsenbeck & Ronquist, 2001) with 4 MCMC heated chains, a chain length of 5 million with a subsampling frequency of 1,000 and a burn-in length of 100,000. A group of *Cyclospora* species (accession numbers: AF111183, AF111185-7), were used to root the phylogenetic tree based on an analysis of the 18S rRNA locus of various *Eimeria* species in previous studies (El-Sherry et al., 2013, Ogedengbe et al., 2015). This analysis was performed on the OTUs from both thresholds (97% and 95% similarity).

5.3.5 Statistical analysis

To compare the outcome of single versus nested PCR for both positive controls and the cohort of BTRW samples that were selected for both single and nested PCR, various parameters were analysed using the non-parametric Kruskal-Wallis test. To compare specificity for *Eimeria* dependent on OTU assignment, *Eimeria* specificity (number of *Eimeria* OTUs/number of total OTUs per sample) was compared between 97% and 95% similarity with a paired t-test. All statistical tests were conducted in Minitab 17.2.1 (LEAD Technologies, Inc., PA, USA).

5.4 Results

5.4.1 PCR and pre-Illumina MiSeq preparation

A total of 117 BTRW faecal samples collected from nine sites were purified and *Eimeria* oocysts were confirmed in 108 samples using bright field microscopy (Table 5.1). All confirmed *Eimeria* positive samples were amplified using single PCR and 58 were suitable for MiSeq sequencing after bands of the correct size were identified by gel electrophoresis and sufficient DNA was present after quantification of amplicons. A subset of 62 samples from the original 117 were selected for nested PCR using criteria that included no amplification in single PCR or they had amplified but their DNA quantity was too low for MiSeq. For MiSeq analysis of the nested PCR amplicons, 11 samples, which included 8 that amplified using single PCR were selected to compare between sample sequences generated by single and nested PCR protocols. The positive control, *E. trichosuri*, was amplified in triplicate in both the single and nested PCRs. With the positive control replicates included, a total of 75 samples were sequenced using Illumina MiSeq.

Table 5.1: Faecal samples from BTRW per site selected for purification. Only samples confirmed to have *Eimeria* oocysts were used in the PCR. Positive samples at the 18S rRNA locus were confirmed to be *Eimeria* with a BlastN search after MiSeq sequencing. KV denotes 'Kangaroo Valley'.

Site	Population Category	# Purified samples	# Samples with confirmed oocysts	18S rRNA positive
Jenolan Caves	Supplemented	11	11	5
KV Mountain	Wild	16	16	5
KV River	Supplemented	34	32	17
KV Creek	Supplemented	10	10	8
KV Enclosure	Captive bred	5	5	5
Nattai	Wild	3	3	3
Square Top	Supplemented	21	14	5
Taronga Zoo	Captive bred	3	3	0
Waterfall Springs	Captive bred	14	14	5

5.4.2 Illumina MiSeq data generation

Using Illumina MiSeq, 28,000,913 sequences were generated from the 75 samples, an average (± SD) of 373,346 ± 101,139 sequences per sample. After initial quality control, 14,837 contigs (comprised of 10,724,040 sequences with an average of 785 ± 313 sequences per contig) were assembled with an average of 199 ± 64 contigs per sample. The average quality score for each sample was highly consistent across the samples; 30.88 ± 5.05 before assembly and 35.04 ± 0.26 for the assembled sequences. After removal of 177 chimeras, alignment with reference sequences and reduction to unique sequences, a total of 992 unique contigs, ~450 bp long, were present. At a sequence similarity threshold of 97%, there were 101 OTUs with up to 11 OTUs per sample. At a lower threshold (95%), there were 58 OTUs with up to 7 OTUs per sample.

5.4.3 Evaluation of the ID similarity within the positive controls and reference sequences

The six positive control replicates (three single and three nested) generated 54 unique contigs. For both the 97% and 95% sequence similarity threshold, all positive control contigs were assigned to a single OTU (OTU 3). The consensus sequence generated from all positive control unique contigs together matched *E. trichosuri* (accession# FJ829323) with 99.8% pairwise identity, with a single nucleotide different at nucleotide 707 of the reference sequence (T replaced a C). The average pairwise identity after alignment with ClustalW between all contig sequences in OTU3 was 98.4% (range 96.7% - 99.5%). There was a single exception of one unique contig from 'Positive Control Single PCR #2' which was assigned to an OTU that matched *E. tenella*.

For the replicates of the positive controls, the single PCR-generated sequences assembled into 560 total contigs while the nested PCR-generated sequences assembled into 819 contigs. From a total of 54 unique contigs after the de-noising algorithms, 22 were from single PCR and 32 from nested PCR. The pairwise identity between the contig sequences within the nested was lower (98.3%) than within the single PCR-generated sequences (98.4%). There was no significant effect of the method on the variables measured for the positive controls (Table 5.2).

To determine which similarity threshold best distinguishes between *Eimeria* species at the 18S rRNA locus, a total of 49 *Eimeria* reference sequences were selected from mammalian and avian hosts (accession numbers in Supplementary table 5.1) and aligned with ClustalW, resulting in an average 94.7% pairwise identity. When the sequences were aligned per host group (bovine, marsupial, poultry, rabbit and rodent), there was an average pairwise identity of 96.98% within each host group. Within the primer-selected region (~445 bp), there were two hypervariable regions within nucleotides 225 to 330 and to a lesser extent within

nucleotides 7 to 111, the latter region mainly variable between bovine and rodent-specific *Eimeria* species. When aligned with the complete reference sequence for 18S rRNA (accession# FJ829320), these regions correspond to nucleotide 405 to 509 and 623 to 728.

Table 5.2: Kruskal Wallis test parameters for comparison between single and nested PCR-generated sequences for the positive controls and the BTRW samples (comparison between the same samples). '*' denotes a significant *p*-value. *Eimeria* specificity was calculated as the proportion of OTUs that matched *Eimeria* in GenBank out of the total OTUs.

	BTRW Samples				Positive Controls			
Statistical parameters	H	d.f.	*p*	Higher median	H	d.f.	*p*	Higher median
All Sequences (before clean-up)	3.19	1	0.074	Single	0.43	1	0.513	Nested
Assembled sequences (after clean-up)	0.54	1	0.462	Nested	0.43	1	0.513	Single
Contigs assembled	0.4	1	0.528	Nested	2.33	1	0.127	Nested
Average sequences per contig	0.01	1	0.916	Nested	2.33	1	0.127	Single
Average Quality for all sequences	6.52	1	0.011*	Nested	1	1	0.317	Same
Average Quality assembled sequences	1	1	0.317	Same	0	1	1	Same
Unique Sequences Generated	0.28	1	0.596	Nested	0.78	1	0.376	Nested
All OTUs at 97% similarity	1.81	1	0.178	Single	1	1	0.317	Same
Eimeria OTUs at 97% similarity	3.72	1	0.054	Single	1	1	0.317	Same
Eimeria specificity at 97% similarity	1.55	1	0.124	Same	1	1	1	Same
All OTUs at 95% similarity	0.11	1	0.744	Same	1	1	0.317	Same
Eimeria OTUs at 95% similarity	0.07	1	0.786	Same	1	1	0.317	Same
Eimeria specificity at 95% similarity	1	1	0.317	Same	1	1	1	Same

5.4.4 Evaluation of generation of unique sequences and OTU assignment in single compared to nested PCRs for BTRW samples

While the single PCR protocol for the BTRW samples before clean-up had a higher average amount of sequences per sample than the nested PCR, there was an overall significantly lower quality score before clean-up ($p = 0.011$, Table 5.2). Post-clean-up, the quality score of the sequences was the same for both methods with a higher average number of assembled sequences per sample for nested PCRs. On average, a higher amount of contigs and unique contig sequences were also generated for the nested amplicons, but not significantly (Table

5.2). When the OTUs were assigned, the single PCR generated on average a higher amount of OTUs per sample than the nested PCR, with a higher amount of *Eimeria*-only OTUs under 97% similarity threshold, though this was not significant ($p = 0.054$, Table 5.2). There was no significant effect of PCR protocol on OTU assignment at 95% similarity, with a similar median between the two PCR protocols. Though there was no significant effect, some differences were observed both in comparing the effect of the PCR protocols on the positive controls and on OTU assignment, mainly at the higher threshold (97%). Due to the generation of different results for the two methods, further analysis only compares single PCR-generated sequences.

5.4.5 Comparison of the BTRW OTU assignment for the 97% and 95% thresholds

A BLAST search using GenBank was performed to match the OTUs to *Eimeria* sequences and of the 58 sequenced samples, 53 samples contained OTUs that matched *Eimeria*. At 97% similarity threshold, there were 59 *Eimeria* OTUs within the BTRW samples, with up to 10 *Eimeria* OTUs per sample. The average pairwise identity after ClustalW alignment within each OTU was 98.4% (range 97.3-99.1%) and the pairwise identity between OTUs was 90.6%. At 95% similarity, there were 28 *Eimeria* OTUs within the BTRW samples. The highest number of *Eimeria* OTUs per sample was 7. Within each OTU, there was an average pairwise identity of 97.7% (range 96.1-98.9%) and the pairwise identity between OTUs was 89.8%. The 97% threshold had a higher number of consensus sequences generated from the OTUs that were >99% similar to each other (7 OTUs) than at the 95% (2 OTUs) so there was poor division between those highly similar OTUs for the 97% threshold. When aligned, the OTU sequences formed a 441 bp consensus sequence for both thresholds. The highest variation in both alignments was located in two ~100 bp regions, from nucleotides 7 to 118 and 220 to 332 of the OTU 1 consensus sequence, which corresponds to nucleotides 406 to 517 and 619 to 731 of the reference *E. trichosuri* sequence (accession# FJ829320). These

hypervariable regions are similar to the variable regions in the alignment for the *Eimeria* reference sequences. A slightly higher proportion of BTRW OTUs were designated *Eimeria* species compared to non-*Eimeria* sequences for 97% similarity (78.77%) than for 95% similarity (77.52) but it was not significant (T-value = 0.36, $p = 0.718$).

5.4.6 Phylogenetic analysis (Bayesian trees)

For both sequence similarity thresholds, Bayesian analysis resulted in most *Eimeria* OTUs forming a highly supported clade with the marsupial-specific *Eimeria* reference sequences (posterior probability > 0.90). Three OTUs from one sample (Kangaroo Valley Mountain) were an exception as they were closely related to *E. maxima* and *E. dispersa*, two poultry-specific *Eimeria* species. For the 97% threshold, there were many OTUs that were closely related and thus there was poorer resolution of relationships (posterior probability for OTU cluster = 0.69) (Fig. 5.2). The phylogenetic tree was better resolved at the 95% threshold as there were fewer closely related OTUs, which clustered with high posterior probability support (>0.90 for most branches, Fig. 5.3). In both trees, the alignment of the single-PCR generated contigs of the positive control, *E. trichosuri*, was included to compare the NGS-generated sequence of a known species to the Sanger sequencing-generated sequence from GenBank.

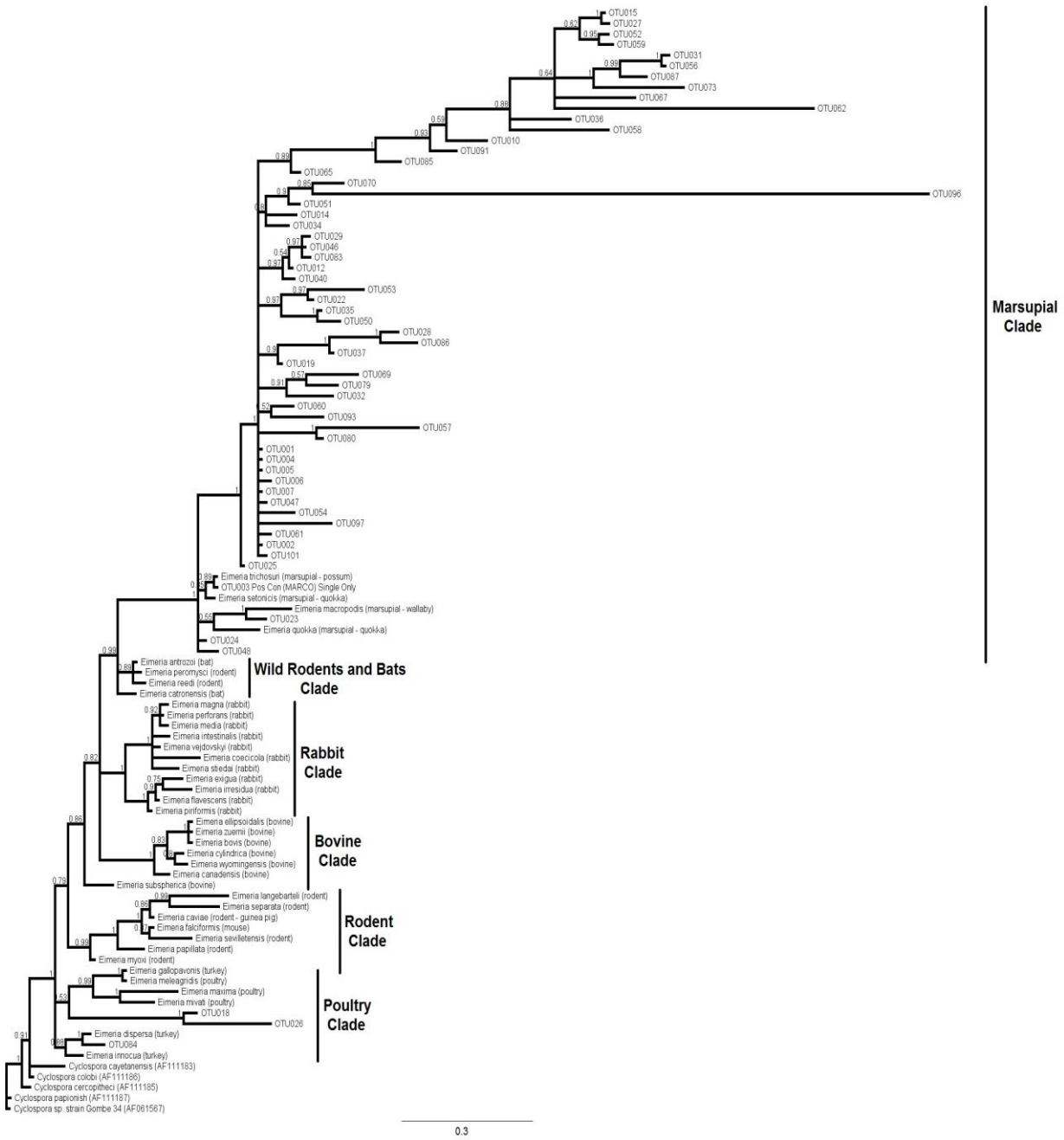

Figure 5.2: Phylogenetic phylogram tree based on Bayesian analysis of the 18S rRNA locus (441 bp) for the 59 BTRW OTUs positive for *Eimeria* and the positive control from *E. trichosuri*. The sample OTUs are compared to the reference *Eimeria* sequences retrieved from GenBank, using the 97% similarity threshold at the OTU assignment step. Posterior probability values are shown at the nodes. The tree was rooted using a group of *Cyclospora* species. The clades indicate the usual host group of the *Eimeria* species. The legend for branch length represents nucleotide substitutions/site.

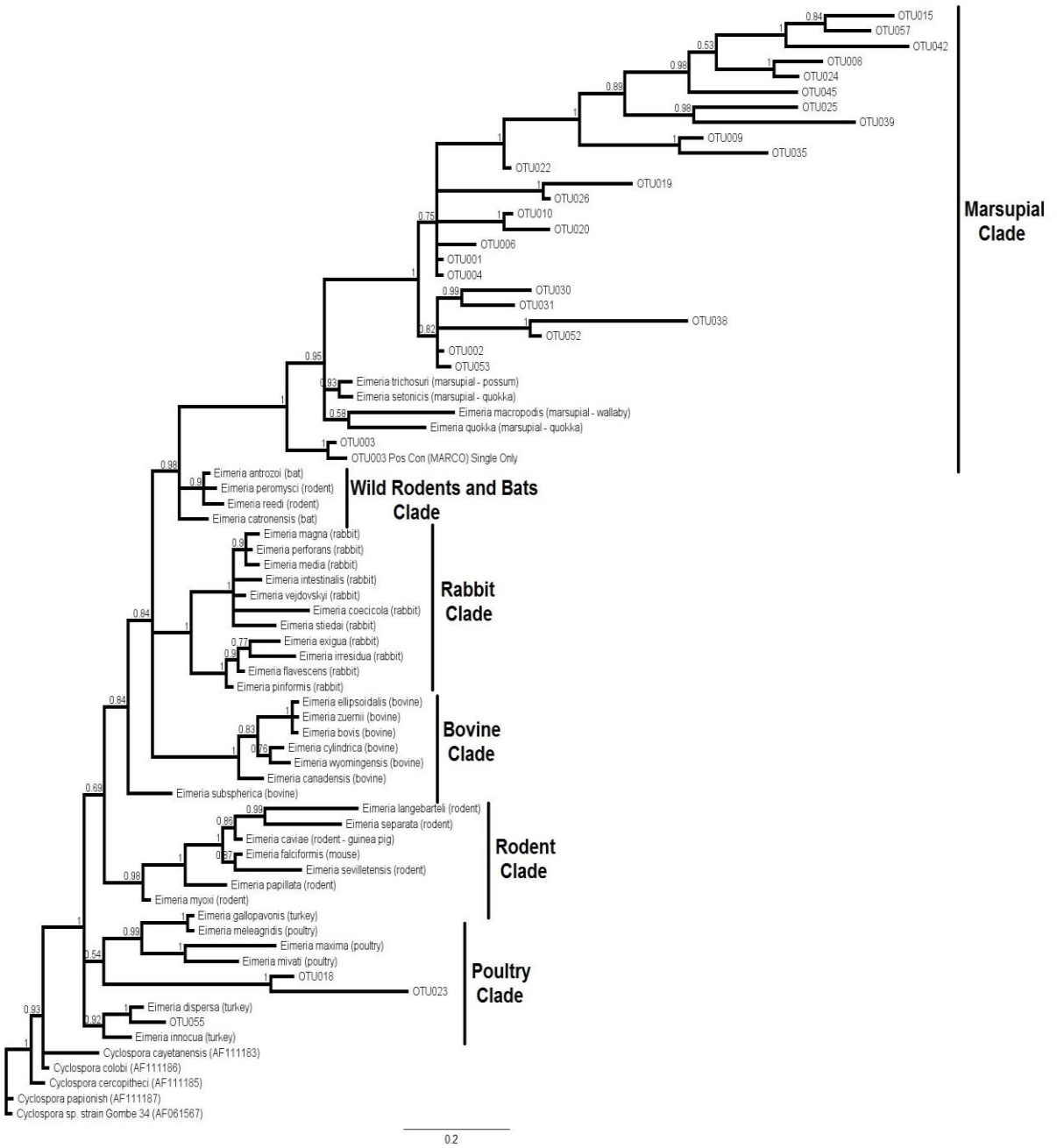

Figure 5.3: Phylogenetic phylogram tree based on Bayesian analysis of the 18S rRNA locus (441 bp) for the 28 BTRW OTUs positive for *Eimeria* and the positive control from *E. trichosuri*. The sample OTUs are compared to reference *Eimeria* sequences retrieved from GenBank, using the 95% similarity threshold at the OTU assignment step. Posterior probability values are shown at the nodes. The tree was rooted using a group of *Cyclospora* species. The clades indicate the usual host group of the *Eimeria* species. The legend for branch length represents nucleotide substitutions/site.

5.5 Discussion

We have developed a simple and cost-efficient approach for the genetic community analysis of *Eimeria*. The PCR and data analysis method was assessed for accuracy of sequencing and sensitivity for *Eimeria*. This study demonstrated that a single PCR protocol was more sensitive than nested PCRs for DNA amplification prior to Illumina sequencing. In addition, OTU assignment was more accurate for assignment of *Eimeria* contigs to *Eimeria* OTUs at a high similarity threshold (97%) but discrimination between OTUs was greater at a lower threshold (95%).

Error rates of sequencing were minimised in this study by the choice of sequencing platform, selection of criteria for quality control (Fig. 5.1), choice of insert and optimization of amplification PCR. The average quality score across all samples prior to clean-up was 30.88, which corresponds to the reported accuracy of Illumina MiSeq, >Q30 (Quail *et al*., 2012). Data clean-up removed 61.7% of sequences, with a further removal of chimeric sequences (18.9%), which is similar to findings in previous studies (Schmidt *et al*., 2013, Bálint *et al*., 2014). The 18S rRNA fragment was selected not only as a hypervariable region but also for a moderate GC content (45.7%) as NGS platforms lose accuracy at GC-extreme areas (Aird *et al*., 2011, Benjamini & Speed, 2012, Quail *et al*., 2012). Amplification bias from the PCR prior to Illumina sequencing was reduced by using a high fidelity DNA polymerase enzyme with a 3′→5′ exonuclease proof-reading activity (Eckert & Kunkel, 1991, Quail *et al*., 2011). Bias can be further reduced through optimisation of the PCR protocol to increase selectiveness while still producing enough DNA product for Illumina sequencing. In the present study, the PCR protocol was optimised by lowering the primer annealing temperature (Aird *et al*., 2011), reducing the Mg^{2+} concentration and decreasing the number of cycles (Eckert & Kunkel, 1991). Applying the developed community analysis method to other

eukaryotes and other genes requires optimisation of the amplification PCR parameters most suitable to the target species and locus.

The purpose of utilising a nested PCR protocol in the amplification PCR was to assess whether this approach increased specificity to *Eimeria* by using an *Eimeria*-specific primer set in the primary reaction of the nested protocol (Zhao & Duszynski, 2001). However, the nested PCR protocol had a higher number of cycles (65 for both reactions combined) compared to the single PCR (45 cycles). There was no significant effect of PCR protocol selection on *Eimeria* specificity, but a significantly higher number of *Eimeria* OTUs were detected in single amplicons compared to the nested amplicons (Table 5.2). To assess accuracy of the PCR protocols, the positive controls were amplified in triplicate for both protocols. The nested protocol generated a higher amount of unique contigs, and although this was not statistically significant, it suggests a lower accuracy and higher error rate in the nested PCRs. The lack of sensitivity to *Eimeria* and lower accuracy indicates there may be higher amplification bias for the nested amplicons without picking up true diversity. This disparity in the nested PCR protocol may be due to the higher amount of cycles (Eckert & Kunkel, 1991). As nested PCRs also have an inherent greater stochastic variation and a greater potential for contamination (Park & Crowley, 2010), utilising a single PCR protocol as amplification PCR in preparation for Illumina MiSeq is recommended, or to limit the amount of cycles as much as possible while optimising the PCR. We observed a limitation of the amplification PCR however as some of the microscopy-confirmed *Eimeria* samples failed to amplify. This disparity may be due to interference of the primer annealing by the overhang adapters required for Illumina MiSeq (Paparini *et al*., 2015, Pawluczyk *et al*., 2015). The reasons of this PCR amplification failure could be further investigated in a future study, such as a larger scale study with different samples of known concentrations of *Eimeria* oocysts to

discern the sensitivity of the amplification PCR. Such a study however would require study hosts with well-studied *Eimeria* strains such as chickens rather than wildlife.

During the OTU assignment stage of data analysis, a sequence similarity threshold most suitable for the study question can be selected. For this study, we assessed two similarity thresholds for OTU assignment. The lower similarity threshold (95%) was selected based on the pairwise identity between selected *Eimeria* reference sequences. Considering that *Eimeria* is usually host specific (Duszynski & Wilber, 1997), the average pairwise identity of *Eimeria* within host groups, 97%, was also considered, hence the selection of the higher similarity threshold. At the 97% threshold, the unique sequences were assigned to a higher number of OTUs, which would allow for greater accuracy for samples that are genetically diverse and a more in-depth examination between OTUs. Also, the slight difference in *Eimeria* specificity (proportion of *Eimeria* OTUs to total OTUs) between thresholds shows that at the high threshold, some unique sequences were assigned to *Eimeria* OTUs while they were assigned to non-*Eimeria* OTUs in the low threshold, lowering the accuracy of OTU assignment at the lower threshold. However, at the high threshold, the consensus sequences for some OTUs were highly similar and thus division between those sequences was poor in the Bayesian tree (Fig. 5.2). In contrast, at the lower (95%) threshold, the OTUs divided into highly-supported separate branches (Fig. 5.3). The contrast between the analyses at different thresholds indicates there is a trade-off between the accuracy of the OTU assignment of *Eimeria* contigs at higher thresholds and greater resolution at lower thresholds. Selection of the similarity threshold depends on the study question posed and the target organism, requiring knowledge at which similarity level the target species can be distinguished and suggesting that during data analysis, it is best to analyse different similarity thresholds. This distinction will become more resolved as more genetic data in databases becomes available for eukaryotes and NGS technology becomes more sensitive, accurate and available to more researchers.

Accuracy of the PCR protocol and data analysis was further tested by analysing replicates of the positive control from the single PCR protocol. The genetic disparity between the replicates (98.4% pairwise identity similarity) may not be solely due to error rates of PCR amplification and sequencing. The 18S rRNA locus of *Eimeria* presents great variation within species and even between paralogs from a single oocyst line, which may be due to its multi-copy nature and the presence of multiple alleles within a single organism (El-Sherry *et al*., 2013). Therefore, this locus may not distinguish well between different *Eimeria* species. However, as only three *Eimeria* species so far have been described in brush-tailed rock-wallabies (Barker *et al*., 1988), the generated sequences may be the same species or very closely related. Thus, the hypervariable 18S rRNA locus is suitable to distinguish between variations within these BTRW *Eimeria* communities. However, depending on the host organism (for instance, a larger scale study of a collection of wildlife samples), there may be greater variation between the sequenced *Eimeria* OTUs. Depending on the study question, a less variable locus may be required such as single copy nuclear genes like cytoplasmic β-actin (*actb*), which can distinguish between genetically close strains even with a short sequence (152 bp) which is highly suitable for Illumina MiSeq (Nolan *et al*., 2015). Other potential nuclear gene targets for which primers are available for short sequences are β-2 microglobulin and tata-binding protein genes (Nolan *et al*., 2015). The mitochondrial cytochrome c oxidase subunit I (COI) has been shown to be a highly reliable marker for distinguishing between *Eimeria* species (Ogedengbe *et al*., 2011, El-Sherry *et al*., 2013) and thus a potential target for barcoding *Eimeria* species sequences from NGS. However, currently available primers tend to amplify regions larger than 500 bp and thus new primers need to be designed for this locus.

As most of the generated OTUs formed a cluster in a highly supported marsupial clade with presumably closely related species (Fig. 5.2 and 5.3), the PCR protocol and Illumina MiSeq

sequencing was accurate for the *Eimeria* sequences. As *Eimeria* is highly host specific and previous studies have failed to cross-infect *Eimeria* species between mammals and birds (Fayer, 1980), it is unlikely the BTRW were infected with poultry-specific *Eimeria* species and thus most likely the sample was contaminated in the environment. The generated sequences had a high *Eimeria* specificity (~80% of OTUs), indicating that the method of purification and amplification PCR with the created primers was highly sensitive to *Eimeria* species. The accuracy and sensitivity of this approach could be further tested in a large scale study utilising *Eimeria* species from different hosts with various known concentrations of oocysts.

We have developed an accurate and sensitive approach that does not require extensive training or a high-powered computer for a genetic analysis of eukaryotic communities. The PCR protocol and data analysis method can be adapted for other eukaryotes by adjusting the preparation method (purification method and choice of primers) and choice of reference sequences from GenBank. Applications for this methodology range from ecological surveys of parasite communities in wildlife to flagging the presence of a known zoonotic or pathogenic parasite within naturally occurring parasite communities. This approach could also be applied to examine parasite community structures within similar hosts from different habitats or treatment regimes, such as administration of anthelmintics or anticoccidials. This methodology allows for a wider range of community analyses in eukaryotes with fewer resources required.

5.6 Acknowledgements

This research was funded by the Australian Research Council, Office of Environment and Heritage and the Australian Museum through the ARC Linkage program, Project ID: LP110200569. The animal ethics approval numbers are AEC 050207/02 and AEC

080728/01. The authors declare that they have no conflict of interest. The authors would like to thank Deborah Ashworth, Todd Soderquist, Celia Thomson, Melinda Norton and Juliet Dingle from Office of Environment and Heritage for collecting the BTRW faecal samples.

5.7 Supplementary material

Supplementary table 5.1: Reference *Eimeria* 18S rRNA sequences from GenBank with the accession numbers, sorted per host group.

Host group	***Eimeria* species**	**Genbank accession number**
Bat	*Eimeria antrozoi*	AF307876
	Eimeria catronensis	AF324213
Bovine	*Eimeria alabamensis*	AB769547
	Eimeria auburnensis	AB769557
	Eimeria bovis	AB769572
	Eimeria bukidnonensis	AB769590
	Eimeria canadensis	AB769602
	Eimeria cylindrica	AB769612
	Eimeria ellipsoidalis	AB769625
	Eimeria subspherica	AB769635
	Eimeria wyomingensis	AB769645
	Eimeria zuernii	AB769655
Marsupial	*Eimeria macropodis*	JQ392574
	Eimeria quokka	KF225636
	Eimeria setonicis	KF225639
	Eimeria trichosuri	FJ829320
Poultry	*Eimeria acervulina*	FJ236372
	Eimeria adeneodei	AF324212
	Eimeria dispersa	HG793041
	Eimeria gallopavonis	HG793042
	Eimeria innocua	HG793045
	Eimeria maxima	FJ236334
	Eimeria meleagridis	HG793039
	Eimeria meleagrimitis	KC305188
	Eimeria mivati	FJ236373
Rabbit	*Eimeria coecicola*	HQ173828
	Eimeria exigua	HQ173829
	Eimeria flavescens	HQ173830
	Eimeria intestinalis	HQ173831
	Eimeria irresidua	HQ173832
	Eimeria magna	HQ173833
	Eimeria perforans	HQ173835
	Eimeria piriformis	HQ173836
	Eimeria stiedai	HQ173837
	Eimeria vejdovskyi	HQ173838

Supplementary Table 5.1 continued

Host group	*Eimeria* species	Genbank accession number
Rodent	*Eimeria alorani*	JQ993659
	Eimeria burdai	JQ993666
	Eimeria cahirinensis	JQ993645
	Eimeria callospermophili	JQ993648
	Eimeria chinchillae	JQ993650
	Eimeria langebarteli	AF311640
	Eimeria myoxi	JF304148
	Eimeria nafuko	JQ993665
	Eimeria papillata	AF311641
	Eimeria peromysci	AF339492
	Eimeria reedi	AF311642
	Eimeria separata	AF311643
	Eimeria sevilletensis	AF311644
	Eimeria vilasi	JQ993653

MOTHUR code:

mothur > summary.seqs(fasta=BTRW.fasta)

mothur > screen.seqs(fasta=BTRW.fasta, maxambig=0, minlength=430, maxlength=460)

#Note: length is estimated around 450 (can check the length of the majority of sequences from the summary in first step)

mothur > unique.seqs(fasta=BTRW.good.fasta)

#Note: Create alignment of *Eimeria* reference sequences for the area you are interested in as fasta file (EimeriaRef.fasta for instance)

mothur > align.seqs(fasta=BTRW.good.unique.fasta, reference=EimeriaRef.fasta, flip=t)

mothur > pre.cluster(fasta=BTRW.good.unique.align, name=BTRW.good.names, diffs=4)

#Note: according to MOTHUR instructions, the diffs are generally set at 1 diff per 100 bp, have ~400 bp

mothur > chimera.uchime(fasta=BTRW.good.unique.precluster.align, name=BTRW.good.unique.precluster.names)

mothur > remove.seqs(fasta=BTRW.good.unique.precluster.align, accnos=BTRW.good.unique.precluster.uchime.accnos)

mothur > dist.seqs(fasta=BTRW.good.unique.precluster.pick.align, calc=nogaps, countends=F, output=square, processors=2)

mothur > cluster(phylip=BTRW.good.unique.precluster.pick.square.dist, method=furthest, cutoff=0.07, hard=t)

#Note: this will generate OTUs at the different cutoffs 0.01 to 0.07 with 0.01 increments)

mothur > bin.seqs(list=BTRW.good.unique.precluster.pick.square.fn.list, fasta=BTRW.good.unique.precluster.pick.align)

mothur > get.oturep(phylip=BTRW.good.unique.precluster.pick.square.dist, list=BTRW.good.unique.precluster.pick.square.fn.list)

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6 Parasites on the hop: captive breeding maintains biodiversity of *Eimeria* communities in an endangered marsupial

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Keywords: *Eimeria*; community analysis; next-generation sequencing; conservation management; parasitology; brush-tailed rock-wallaby

Published in: *Biological Conservation*, 200, 17-25

DOI: 10.1016/j.biocon.2016.05.019

6.1 Abstract

The complex parasite communities of animals contribute to biodiversity, yet the conservation strategies that aim to preserve individual threatened species often overlook their parasite communities. Deeper understanding of parasite communities and how they are affected by conservation management is important to the ultimate success of biodiversity conservation. Here we examine the dynamics between the coccidian parasite *Eimeria* and the threatened brush-tailed rock-wallaby (*Petrogale penicillata,* BTRW) to determine how parasite communities respond to conservation management practices of captive breeding and translocation. Three BTRW population categories (wild, captive bred or supplemented) were analysed for *Eimeria* prevalence and infection intensity and a genetic analysis of the *Eimeria* communities. *Eimeria* prevalence was 92.3% in 117 faecal samples. DNA amplicons from purified oocysts were sequenced with the Illumina MiSeq platform and the resulting sequences assigned to 28 *Eimeria* operational taxonomic units (OTU). Pairwise identity between OTUs was 89.9% and 25 of the *Eimeria* OTUs formed a highly supported phylogenetic clade with marsupial specific *Eimeria* species, indicating strong host specificity and genetic diversity within *Eimeria* in BTRWs. Supplemented populations had the greatest OTU diversity with eleven unique OTUs and had a greater overlap with captive bred (9 shared OTUs) versus wild populations (6 shared OTUs). There was no significant effect of population category on infection intensity ($p = 0.965$), OTU composition ($p = 0.51$) or OTU richness ($p = 0.490$), suggesting that *Eimeria* community structure is maintained under the management processes applied to the BTRW. Our approach can be applied to other parasite communities in hosts under conservation management.

6.2 Introduction

Communities of parasites and their association with free-living organisms form an integral component of biodiversity (Gómez & Nichols, 2013). The interaction between the host and its parasites drives an antagonistic coevolution that may change host behaviour, biology and genome, thus increasing biodiversity of both host and parasite (reviewed in Paterson & Piertney, 2011). Conservation management aims to maintain or increase populations of threatened species through management strategies such as captive breeding and translocation of individuals into diminished populations (Griffith *et al*., 1989, Meffe *et al*., 2006, NRMMC, 2010). However, parasite communities have often been ignored in conservation management plans (Gómez & Nichols, 2013) or only considered from the perspective of disease management (Horan & Melstrom, 2011). This oversight occurs even though conservation strategies may lower parasite diversity (Moir *et al*., 2012). Furthermore, conservation strategies often involve handling of threatened species by humans and introduction of individuals to possibly atypical environments. Such factors may result in the introduction of novel parasites (Cunningham, 1996), induce stress in the host and/or lower immunity (Moberg, 1985), creating an imbalance in the relationship between host and specialist parasites (Moir *et al*., 2012). Specialist parasites are also constrained by geographical range due to host specificity or requirement of particular environmental conditions for transference between hosts (Thompson, 1999, Poulin *et al*., 2012). A greater understanding of how parasite communities of threatened wildlife respond to conservation management strategies may allow for greater success in maintaining biodiversity.

The protozoan *Eimeria* (Apicomplexa: Eimeriidae), one of the causative agents of coccidiosis, is considered a specialist parasite due to its high host specificity (Duszynski & Wilber, 1997), though some species may infect closely-related and sympatric hosts (Barker *et al*., 1988a). Identification of *Eimeria* species is traditionally based on the morphological

features of the sporulated oocyst containing the infective sporozoites (Duszynski & Wilber, 1997). This method can be complicated by limited variation in morphological traits between species (Long & Joyner, 1984, Zhao *et al*., 2001) or variation within the same species (Joyner, 1982). The issues associated with species identification using morphology have resulted in approaches using a combination of morphological and molecular techniques (reviewed in Blake, 2015). Identification using molecular analyses has mainly been conducted on commercially significant *Eimeria* species from domestic hosts such as chickens (Chapman & Shirley, 2003) and cattle (Daugschies & Najdrowski, 2005). More recently molecular methods have been applied to wildlife species for both identification and phylogenetic inference (Zhao *et al*., 2001, Power *et al*., 2009, Afonso *et al*., 2014, Austen *et al*., 2014). However, genetic analysis of *Eimeria* in naturally occurring hosts is often complicated by concurrent infections of different *Eimeria* species leading to difficulties with assigning sequence data to described *Eimeria* species (Yang *et al*., 2012). There is also very little information of the community structure of *Eimeria* in any host.

Next-generation sequencing (NGS) has been widely applied to assess the community composition of bacteria (Faust & Raes, 2012) with minimal applications to eukaryotes (Bik *et al*., 2012). Recently, NGS has been applied to microbial parasites important to human health such as *Plasmodium* (Carlton *et al*., 2013) and *Cryptosporidium* (Paparini *et al*., 2015). For *Eimeria*, NGS has been used for whole genome evaluation and transcriptomics (Matsubayashi *et al*., 2013, Heitlinger *et al*., 2014, Hong *et al*., 2014, Reid *et al*., 2014). Few studies have applied NGS to examine community structure of parasites, such as examining the community structure of nematodes in different populations of captive kangaroos (Lott *et al*., 2015).

While coccidiosis is a significant disease in commercial industries such as meat and poultry production (Chapman *et al*., 2013), *Eimeria* is generally thought to be non-pathogenic in free-

ranging wildlife (Hum *et al*., 1991, Debenham *et al*., 2012, Winternitz *et al*., 2012). However, *Eimeria* pathogenicity can increase with higher infection intensity (Hum *et al*., 1991, Daugschies & Najdrowski, 2005) and external stressors (Fayer, 1980). For example, in Australian wildlife, reports of coccidiosis are rare with disease primarily reported for juveniles in captivity or being hand-reared (Munday, 1988, Hum *et al*., 1991). A single study has reported coccidiosis in wild kangaroos (*Macropus giganteus*) confined by flood and experiencing limited food resources (Barker *et al*., 1972). Conservation actions may drive the risks for coccidiosis particularly due to host density and stressors which may vary between captive, translocated and wild animals (Cunningham, 1996). Hence we aim to investigate differences in *Eimeria* prevalence and infection intensity between different populations of animals undergoing conservation management, as well as investigate the genetic diversity and community structure of *Eimeria* in these individuals.

The brush-tailed rock-wallaby (BTRW, *Petrogale penicillata*, Marsupialia: Macropodidae) is a 6-8 kg marsupial currently subject to conservation management involving captive breeding and translocation of individuals between captive and wild populations (Eldridge, 2008, Menkhorst & Hynes, 2010). The BTRW has declined significantly in the last 150 years (Lunney *et al*., 1997) and is listed as 'near threatened' on the International Union for Conservation of Nature (IUCN) Red List (IUCN, 2015) and 'Endangered' in New South Wales, Australia (NSW *Threatened Species Conservation Act* 1995). Three *Eimeria* species have been previously described in BTRWs with information on these species limited to morphological descriptions (Barker *et al*., 1988b). BTRW populations experiencing varying levels of conservation management were selected for this study. Populations sampled comprised those housed in captive breeding facilities, wild sites supplemented with individuals from captive facilities, and undisturbed wild sites. Comparisons between *Eimeria* prevalence, infection intensity and the genetic community structure using NGS was

determined for each population type to investigate responses of parasite communities to management.

6.3 Methods

6.3.1 Sample collection

Populations of the BTRW are fragmented across their range in eastern Australia. Contact between populations is now rare since BTRW inhabit patches of rocky outcrops and cliffs in isolated mountain areas (Browning *et al*., 2001, Eldridge & Close, 2005). Nine populations from different locations in New South Wales, Australia, were used in this study (Suppl. Table 6.1, 6.2). The sites were divided into three population categories depending on management activities: wallabies bred in captivity across several generations in captive breeding facilities (captive bred), free ranging wallabies (wild) and free ranging populations supplemented with captive bred wallabies (supplemented), summarised in Table 6.1 with background information for each site supplied in supplementary Table 6.2. Kangaroo Valley (KV) Enclosure is an enclosed area where captive bred animals were kept for a 'hardening' period prior to release into free ranging population. No anticoccidials or anthelmintics were administered to individuals prior to release or movement between sites. Faecal samples were collected opportunistically over a period of five years between March 2009 and December 2014 during routine site management by staff from Office of Environment and Heritage, NSW, Australia (Suppl. Table 6.1). After collection, samples were stored in plastic vials with silicon beads and kept at 4°C until further processing.

Table 6.1: Faecal samples collected from nine BTRW in sites in NSW and selected for oocyst purification. A positive result for *Eimeria* was confirmed at the 18S rRNA locus after PCR amplification. KV denotes Kangaroo Valley.

Site	Population Category	Sucrose purified samples	18S rRNA PCR positive
Jenolan Caves	Supplemented	11	5
KV Mountain	Wild	16	5
KV River	Supplemented	34	17
KV Creek	Supplemented	10	8
KV Enclosure	Captive bred	5	5
Nattai	Wild	3	3
Square Top	Supplemented	21	5
Taronga Zoo	Captive bred	3	0
Waterfall Springs	Captive bred	14	5

6.3.2 Parasite purification and DNA extraction

Oocysts from BTRW faecal samples (n = 117) collected at nine sites (Table 6.1) were purified using a sucrose flotation technique (Truong & Ferrari, 2006), modified for a smaller faecal volume (Vermeulen *et al*., 2016 = Chapter 5). Purified samples were examined for the presence of *Eimeria* oocysts using bright-field microscopy (Olympus Corporation, Tokyo, Japan) at 100x magnification and DNA extracted from samples found to be positive. Prior to DNA extraction oocyst walls were disrupted by combining a solution of suspended purified oocysts (50 µL) with glass beads (100 mg, 425-600 µm, Sigma-Aldrich, MO, USA) and vortexing for 1 min (Ogedengbe *et al*., 2011). DNA was then extracted using PrepGEM (ZyGEM Corporation, Hamilton, New Zealand) following a previously developed protocol (Power *et al*., 2009). The extracted DNA was stored at -20°C.

6.3.3 Infection intensity of *Eimeria*

To determine oocyst shedding and infection intensity, sucrose flotation purified samples positive for *Eimeria* and with sufficient faecal material (~0.5 g) were subject to quantitative

faecal flotation. The number of *Eimeria* oocysts per gram of faeces (OPG) was determined using zinc sulphate flotation (Daugschies *et al*., 1999) and the McMaster chamber method (Henriksen & Aagaard, 1976) using bright-field microscopy at 100x magnification.

6.3.4 Amplification PCR and Illumina MiSeq sequencing

The 18S rRNA locus was amplified using a previously described PCR protocol on the DNA extracted from the purified *Eimeria* oocysts. The primers 365F and 872R with adapters compatible with Illumina MiSeq index and sequencing were used to amplify a ~500 bp fragment (Vermeulen *et al*., 2016 = Chapter 5). *Eimeria trichosuri* DNA was used as the positive control for all PCRs. PCR amplicons were resolved using 2% gel electrophoresis and subsequently purified using the QIAquick PCR purification kit following manufacturer's instructions (QIAGEN, Hilden, Germany). DNA concentrations were measured using Qubit® 2.0 Fluorometer (Life Technologies, Mulgrave, VIC, Australia) and standardised to 5-20 ng/µl. Using the Nextera XT Index kit (Illumina, San Diego, USA) genomic libraries were prepared and sequencing performed in 300 bp paired-end reads on the Illumina MiSeq sequencing platform (Ramaciotti Centre for Gene Function Analysis, University of New South Wales, Sydney, Australia). All sequences generated in this study were deposited in the NCBI sequence read archive (SRA) under the study accession number SRP062125.

6.3.5 Data analysis

The post-NGS data analysis followed a protocol previously described by Vermeulen *et al*. (2016 = Chapter 5). Initial quality control (trimming of adapter sequences and terminal nucleotides, removal of low quality bases) and de novo assembly of contigs from paired-end reads with a minimum match percentage of 99% (with removal of small (<100 bp) and low frequency (<100 sequences) reads) were conducted in SeqMan NGen (DNASTAR, Madison, USA). Contigs were subsequently imported into MOTHUR (Schloss *et al*., 2009) for correct

orientation using an *Eimeria trichosuri* reference sequence (accession # FJ829320) and then further denoised through various quality control algorithms such as pre-clustering, unique sequence generation and removal of chimeric sequences using the Chimera.bellerophon function (Schloss *et al*., 2009). Unique sequences were assigned to operational taxonomic units (OTUs) using pairwise sequence distances with free end gaps and subsequently clustered with the furthest neighbour algorithm at the minimum identity threshold of 95%. To identify *Eimeria* sequences, a consensus sequence for each OTU was compared to reference sequences in GenBank (NCBI) using the BlastN function in Geneious (Biomatters LtD, New Zealand). OTUs were assigned to *Eimeria* based on a high sequence identity (>85%) to a reference *Eimeria* sequence and 100% query coverage, as less than 100% could indicate an incorrectly assembled contig and was thus treated as suspicious and left out of the analysis (Suppl. Table 6.3).

6.3.6 Phylogenetic analysis

Bayesian Markov Chain Monte Carlo analysis, using a general time reversible model with a gamma distribution with four rate categories, was performed using Mr. Bayes 3.2.2 (Huelsenbeck & Ronquist, 2001) in Geneious 7.1.9 (Biomatters Ltd., New Zealand), with 4 MCMC heated chains, a burn-in length of 100,000 and a chain length of 5 million with a subsampling frequency of 1,000. A Bayesian tree was constructed of the BTRW OTUs determined to belong to the genus *Eimeria* (Suppl. Table 6.3) and 38 reference *Eimeria* sequences (36 mammalian specific and 2 poultry specific *Eimeria* species based on a match with three non-marsupial *Eimeria* isolates) retrieved from GenBank, with a group of *Cyclospora* species (accession numbers: AF111183 and AF111185-AF11187) used to root the phylogenetic tree (El-Sherry *et al*., 2013).

6.3.7 Statistical analysis

To test for statistically significant associations between different variables for the *Eimeria* populations, various statistical analyses (Spearman rho correlation, Kruskal-Wallis non-parametric test) were performed using MiniTab 17.2.1 (LEAD Technologies, Inc., PA, USA). The rarefaction curves were generated in R v. 3.2.2 (R Core Team, 2015) using the vegan package (Oksanen *et al*., 2015). Relative OTU prevalence was calculated as frequency of occurrence of each OTU within the *Eimeria* communities of each population category divided by the total amount of samples within the category, expressed as percentage of total samples. To compare OTU composition (based on presence or absence of each OTU in each sample) between *Eimeria* communities, *Eimeria* OTU assignment to samples was analysed with the Jaccard resemblance coefficient using PRIMER Version 6.1.11 (Plymouth Marine Laboratories). OTU composition among samples within sites and population category was analysed using hierarchal clustering in PRIMER. The significance of the effect of *Eimeria* infection intensity, date of collection, site and population category on OTU composition was tested with an analysis of similarity (ANOSIM) in PRIMER.

6.4 Results

6.4.1 *Eimeria* prevalence and infection intensity

Eimeria oocysts were detected in 108 of 117 sucrose purified samples (92.3%). Oocyst shedding for the 84 samples examined using faecal flotation ranged from 103 to 188,820 OPG (mean = 13,891 OPG). The highest mean OPG (± standard deviation) was found in the supplemented population, 15,366 ± 31,436 (median = 5668), with the lowest in the wild population, 10,208 ± 11,917 (median = 6549). The mean captive bred OPG was 12094 ± 17656 (median = 4837). Variation in infection intensity was not significant between population categories (Kruskal-Wallis H = 0.07, d.f. = 2, p = 0.965) and between individual

sampling sites (H = 8.61, d.f. = 7, p = 0.282). A summary of the results of the OPG (means, standard deviations and medians) for each site was included in Supplementary Fig. 6.4.

6.4.2 Amplification PCR and Illumina MiSeq data output

The 108 samples confirmed to contain *Eimeria* oocysts were subsequently used for PCR. Amplification at the 18S rRNA was successful for 58 samples based on correctly-sized band on gel electrophoresis and DNA quantification which were then sequenced using the Illumina MiSeq. MiSeq generated 8,097,021 sequences after quality control, assembled into 10,975 contigs with an average of 810 contigs per sample. OTU assignment using 95% similarity threshold resulted in a total of 56 OTUs. Following the matching of consensus sequences of each OTU to reference *Eimeria* sequences from GenBank, 28 OTUs were found to match the genus *Eimeria* (Suppl. Table 6.1 and 6.3). These *Eimeria* OTUs were present in 53 samples out of 58 sequenced samples. The contig length for each OTU was 441 bp with an average pairwise identity of 89.9% between the consensus sequences (range 77.4% - 100%), while the similarity of unique sequences within each OTU was on average 97.7% (range 96.1 - 98.9%).

6.4.3 Phylogeny of *Eimeria* in BTRW samples

In the Bayesian tree of the *Eimeria* OTUs and *Eimeria* reference sequences, most of the sampled BTRW *Eimeria* OTUs (25/28) formed a highly supported clade with known marsupial *Eimeria* species (posterior probability > 0.90). One sample (KV Mountain, wild) formed the exception by clustering three OTUs with two poultry specific *Eimeria* species, *E. dispersa* and *E. maxima* (Fig. 6.1). Within the marsupial clade, there was a cluster of OTUs assigned to supplemented and captive bred samples only. For further analysis of OTU biodiversity, only the marsupial-specific *Eimeria* OTUs were included.

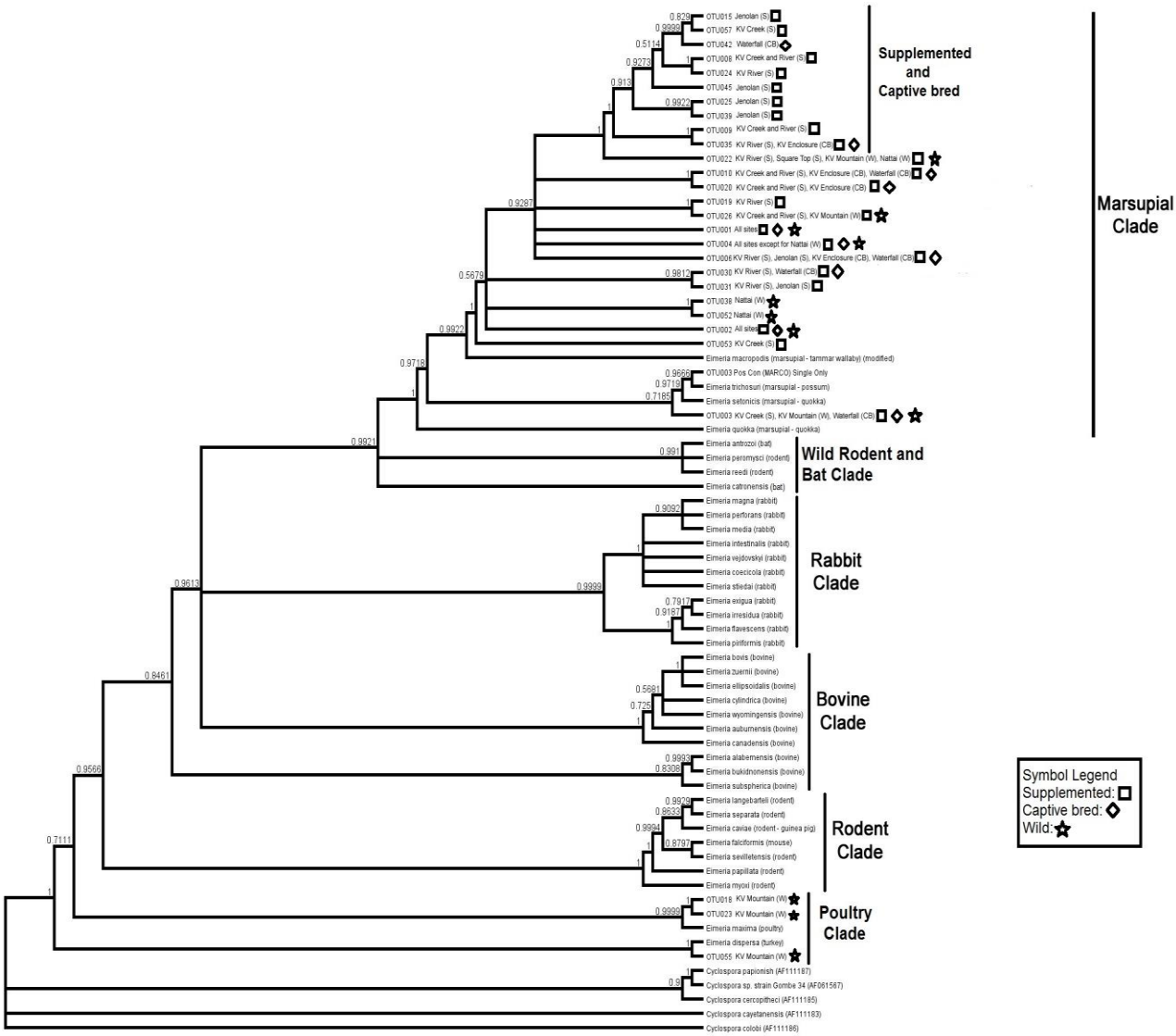

Figure 6.1: Phylogenetic relationships following Bayesian analysis of 441 bp of the 18S rRNA locus for the 28 *Eimeria* OTUs identified in the BTRW samples along with the positive control *E. trichosuri*. The *Eimeria* OTUs were compared to reference sequences for various mammalian and two avian specific *Eimeria* from GenBank. The collection site of each OTU is indicated, followed by a letter code denoting the BTRW population category: W = wild, S = supplemented and CB = captive bred. Specific host group clades are indicated. Values at the nodes are posterior probability values. The tree was rooted using a group of *Cyclospora* species.

6.4.4 OTU richness and composition across sites and population categories

On average, three *Eimeria* OTUs were detected in each sample, with a maximum of seven OTUs identified in one sample from a supplemented site (KV River). Six samples had only one *Eimeria* OTU, five from supplemented sites KV River (3) and KV Creek (2), and one wild sample (KV Mountain). Site and population category had no significant effect on OTU richness, defined as number of OTUs per sample (Kruskal-Wallis, H = 7.82, d.f. = 7, p = 0.349 and H = 1.43, d.f. = 2, p = 0.490 respectively). Rarefaction analysis indicated that no population category had been completely sampled for OTU richness (reached an asymptote), but within the range of common sampling effort, there was no significant difference in richness, as indicated by the 95% confidence interval of the supplemented samples (Fig. 6.2).

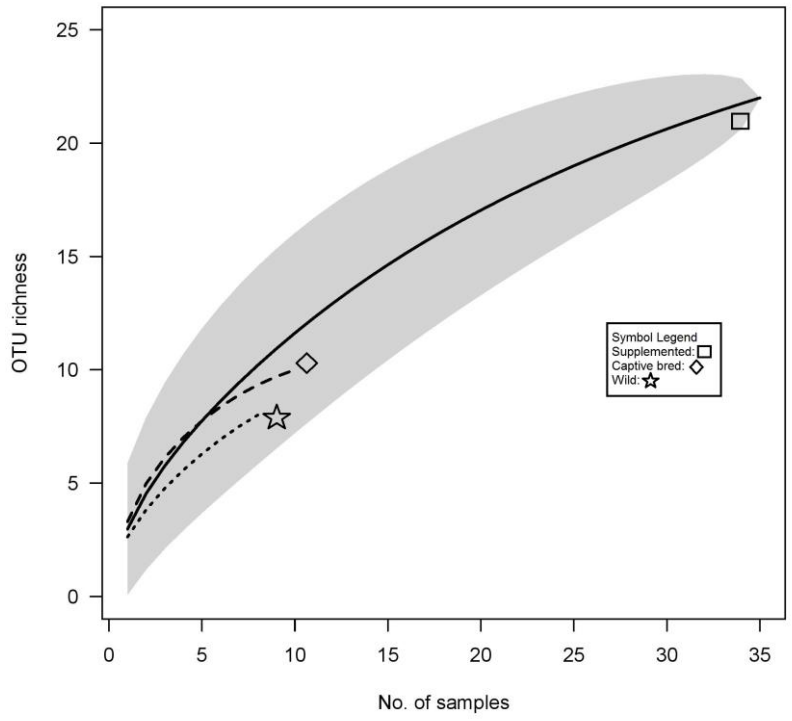

Figure 6.2: Rarefaction curves of OTU richness (expected number of OTUs) for a given number of samples. Solid line represents supplemented, dashed line is captive bred and dotted line is wild. The light grey polygon shows an approximation of the 95% confidence

interval (two times standard deviation) for the supplemented samples. For the purpose of clarity, confidence interval was only given for the supplemented samples. The samples represented in this figure only include the samples containing OTUs that were part of the marsupial *Eimeria* clade in the Bayesian analysis.

There was no significant relationship between infection intensity and OTU richness (Spearman rho correlation test, $\rho = 0.220$, $p = 0.242$) or OTU composition (infection intensity in bins, ANOSIM, R = -0.033, $p = 0.61$). There was no significant effect on OTU composition from population category (ANOSIM, R = -0.007, $p = 0.51$) or site (ANOSIM, R = -0.002, $p = 0.471$).

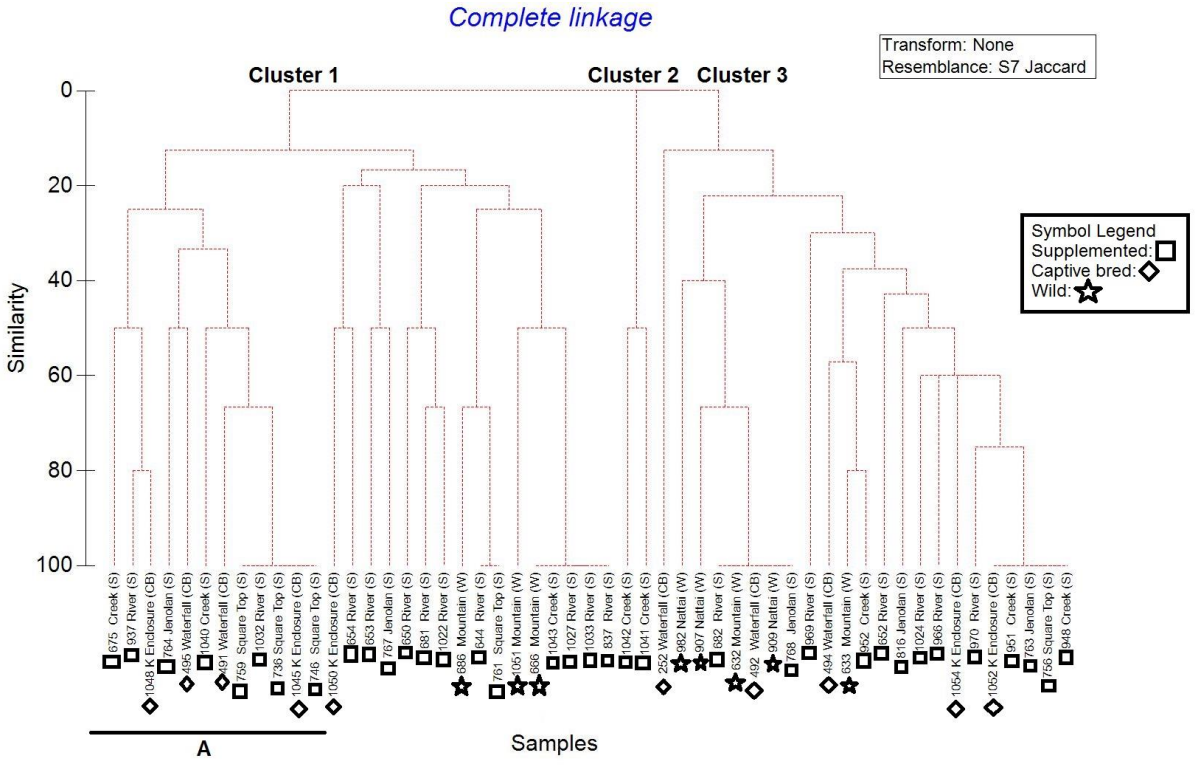

Figure 6.3: Hierarchal clustering of each *Eimeria* community based on the Jaccard resemblance coefficient. The locations and BTRW population categories are indicated after the site name (W = wild, S = supplemented and CB = captive bred). The three major clusters are indicated by number while the cluster containing only captive bred and supplemented

isolates is indicated with ‘A’. The scale indicates similarity of OTU richness between samples based on presence or absence of OTUs. The OTUs represented are only the OTUs that were part of the marsupial *Eimeria* clade in the Bayesian analysis.

Hierarchal cluster analysis was based on the similarity profile of OTUs present in each sample (OTU richness). The hierarchal cluster split into three clusters at 0% similarity, with cluster 2 only containing two samples from a supplemented population, KV Creek (Fig. 6.3). Cluster 1 divided into two main groups at the ~12% similarity level (see scale on Fig. 6.3), one of which consisted of only supplemented and captive-bred samples (denoted A).

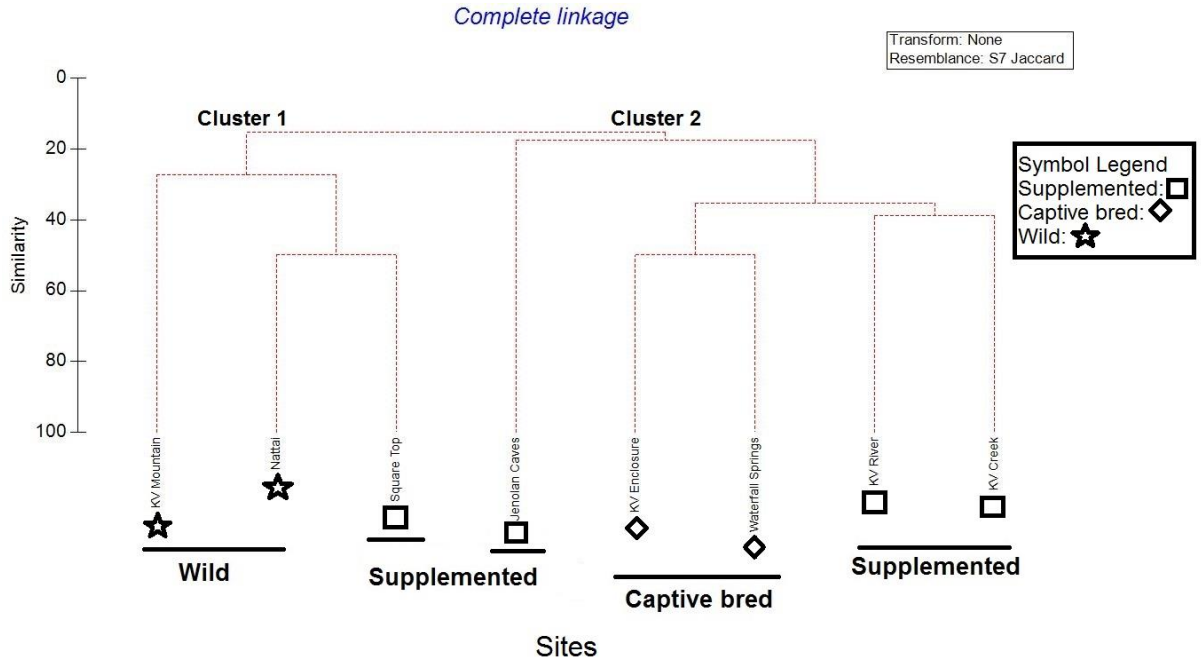

Figure 6.4: Hierarchal clustering based on the Jaccard resemblance coefficient where each *Eimeria* community was grouped within their site location, with BTRW population category indicated underneath the site name. The two major clusters identified are indicated by number. The scale indicates similarity of OTU richness between sites based on presence or absence of OTUs. The OTUs represented are only the OTUs that were part of the marsupial *Eimeria* clade in the Bayesian analysis. KV denotes ‘Kangaroo Valley’.

When the samples were grouped per site, the hierarchal cluster analysis of OTU richness identified 2 main clusters dividing at ~18% similarity (Fig. 6.4). Cluster 1 contained the wild sites with the exception of Square Top, and Cluster 2 contained only supplemented and captive bred samples. Jenolan Caves appears the most different from other sites, dividing from the other samples in cluster 2 at ~20% similarity.

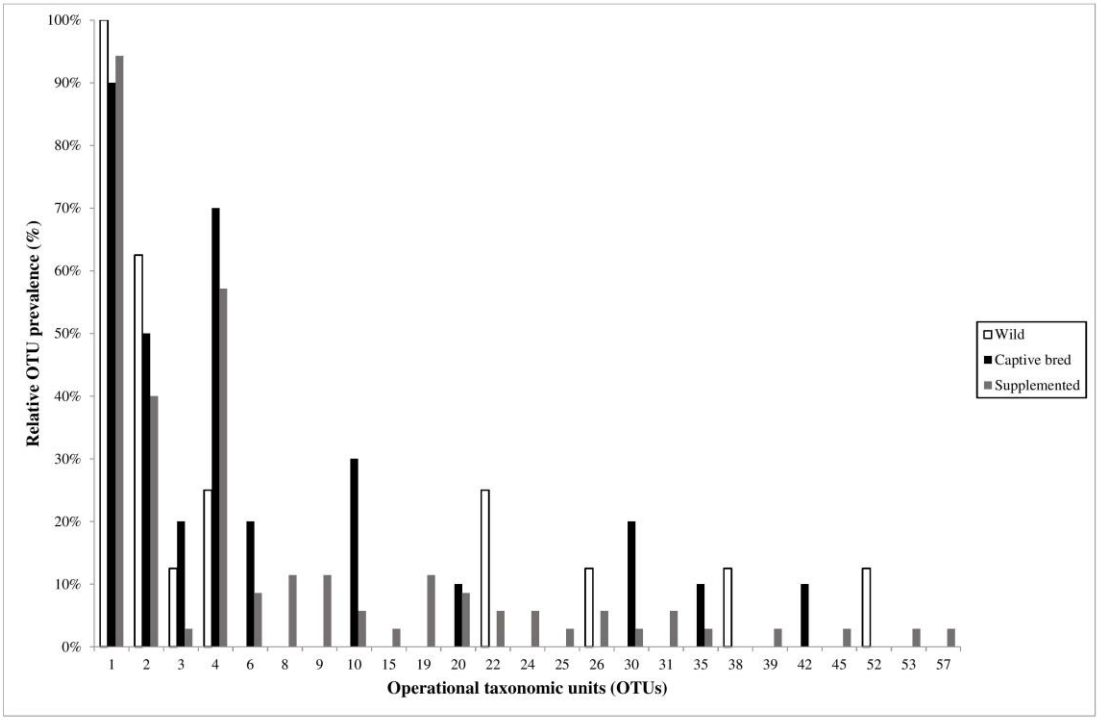

Figure 6.5: Relative prevalence of each marsupial-matching *Eimeria* OTU within each BTRW population category. OTU assignment sorted the unique sequences hierarchally from the highest number of sequences in OTU 1 to the lowest in OTU 57 and non-marsupial *Eimeria* OTUs (poultry-specific OTUs) were excluded in the analysis. OTU 3 has a lower prevalence due to the presence of positive control sequences previously discussed in Vermeulen *et al*. (2016).

Relative OTU prevalence revealed further distinctive patterns between population categories (Fig. 5). The OTUs with highest prevalence (OTU 1-4) were found in all three population categories but OTUs with lower prevalence (OTU 6 and higher) were found in either only one or two categories. The highest diversity of OTUs (11 unique OTUs) was observed in supplemented populations. One OTU was found only in captive bred samples (OTU 42) and five OTUs were unique to wild samples. There were five OTUs present in a combination of captive bred and supplemented sites only and two OTUs in a combination of supplemented and wild sites only.

6.5 Discussion

Conservation management strategies have the potential to alter host-parasite relationships. Here we used a community analysis approach with NGS to examine *Eimeria* communities from different BTRW populations, as well as examining infection intensity and prevalence. However, this may not be the true prevalence of *Eimeria* due to the possibility of pseudo-replication from opportunistic sampling which needs to be taken into account when interpreting these data.

The approaches of PCR and microscopy applied in the study varied in their ability to identify *Eimeria* presence. Not all tested samples were positive by PCR and the combination of sucrose flotation and microscopy demonstrated higher parasite presence. While PCR is often more sensitive for detecting *Eimeria* than microscopy (Austen *et al*., 2014), only 58 out of 108 microscopy-confirmed samples produced enough DNA product in the amplification PCR for subsequent sequencing analysis. Failure to amplify some BTRW samples may be due to the addition of overhang adapters interfering with the efficiency of primer annealing in the amplification PCR (Pawluczyk *et al*., 2015, Paparini *et al*., 2015) or presence of PCR inhibitors. As the amplification PCR is required as a first step for Illumina MiSeq sequencing,

PCR failure is potentially a significant issue for accuracy and sensitivity of sequencing. Therefore, further investigation is required and a future, more extensive study may integrate quantitative analysis such as qPCR to further optimise the amplification PCR, as well as investigate the choice of primers and loci, depending on the organism to be investigated (Pawluczyk *et al*., 2015).

Eimeria was almost ubiquitous in all BTRW populations with a prevalence of 92.3% (108/117). This observed high prevalence in BTRWs is congruent with other studies, where the prevalence of *Eimeria* in rock-wallabies ranged from 25% to 100%, with an average of 66% (Barker *et al*., 1988b, O'Callaghan *et al*., 1998). The high prevalence among BTRW populations appears to concur with the previous observation that *Eimeria* parasites generally have low pathogenicity in marsupials unless they are under stress (Barker *et al*., 1972). Oocyst shedding was not affected by BTRW population category, suggesting conservation management had no significant impact on infection intensity of *Eimeria*. While no significant effect of population category was detected on oocyst shedding, variation was seen in *Eimeria* biodiversity and community structure among the managed and free ranging populations.

High genetic diversity was observed within BTRW *Eimeria* with 509 unique *Eimeria* sequences generated with a high range of pairwise identity (76.9% - 99.1%) and being assigned to 28 *Eimeria* OTUs. The observed diversity of *Eimeria* OTUs in BTRW may represent multiple infections of *Eimeria* which are commonly seen amongst infected hosts (Barker *et al*., 1989, Jeanes *et al*., 2013, Austen *et al*., 2014), including the BTRW (Barker *et al*., 1988b). Only three *Eimeria* species have previously been described in BTRW (Barker *et al*., 1988b) yet the high number of *Eimeria* OTUs and their range in pairwise identity suggests there are more species. However, single species infections in the BTRW cannot be ruled out. The 18S rRNA locus of *Eimeria* is highly variable within species (Ogedengbe *et al*., 2011, Poplstein & Vrba, 2011, Vrba *et al*., 2011), and an ~97% pairwise identity has been

described between paralogs of the same *Eimeria* species (El-Sherry *et al*., 2013). Heterozygosity in the 18S rRNA locus for *Eimeria* from another macropodid marsupial, the tammar wallaby (*Macropus eugenii*), has also been reported (Hill *et al*., 2012). To determine if only a single species or multiple species are present in the BTRW, a repeat study could be performed using samples with high genetic diversity by using a locus that can better distinguish *Eimeria* species e.g. the mitochondrial cytochrome c oxidase subunit I (COI) gene (Ogedengbe *et al*., 2011, El-Sherry *et al*., 2013). However, new primers will need to be designed for this locus. A more traditional approach would be to identify species by combining the molecular data of samples containing a single OTU with morphological data of oocysts through microscopy.

To unravel the genetic complexity for samples that have a single OTU, subsequent morphological analysis of the oocyst may be able to identify the species using previous descriptions of *Eimeria* species (Barker *et al*., 1988b, Duszynski & Wilber, 1997). This combined approach will improve understanding of *Eimeria* diversity in both captive and wild marsupials, where few studies to date have included genetic data (Power *et al*., 2009, Hill *et al*., 2012, Austen *et al*., 2014). Given that NGS has not been previously applied to *Eimeria,* and that the method allows for sensitive analysis of the entire *Eimeria* population, further studies on *Eimeria* communities are required to understand how the genetic diversity relates to species diversity.

Phylogenetic analysis of the NGS sequences supported strong host specificity for the majority of the *Eimeria* OTUs which formed a highly supported clade with other marsupial specific *Eimeria* species. An exception was three OTUs from a single sample from a wild site that grouped with poultry *Eimeria* species. It is unlikely that *Eimeria* species common in poultry are causing infection in BTRWs as these parasites have very high host specificity (Duszynski & Wilber, 1997). Attempts to cross-infect *Eimeria* species between evolutionary removed

hosts such as mammals and birds have previously failed (reviewed in Fayer, 1980). The BTRW faecal sample returning sequences matching avian-specific *Eimeria* may have been contaminated with wild bird faecal matter.

OTU richness between samples had distinguishing patterns between population categories. Most of the overlap in OTU richness observed in the various analyses was between captive bred and supplemented populations. However, unless individuals were tested prior to release and observed post-release in the wild, such as through tagging and trapping, we cannot say with certainty that the same OTUs in the same individuals are maintained from captive facilities into supplemented populations. The similarity between OTUs in captive and supplemented populations still suggests that some *Eimeria* OTUs are unique to BTRWs bred in captivity due to the isolation of captive bred wallabies from wild populations. Highly host specific parasites are specialised to host populations, particularly if they are isolated or fragmented (Thompson, 1999, Froeschke *et al*., 2013). *Eimeria* also transfers between hosts using the faecal-oral route through the environment and thus can be limited to a geographical location (Thompson, 1999, Marquardt *et al*., 2000). The greatest unique OTU diversity was observed in the supplemented sites, where a wild population was supplemented with captive bred animals. Future studies could observe such mixed host populations over a longer period of time to observe whether gene flow introduced unique OTUs that remain in the parasite population and increase its genetic diversity (Hague & Routman, 2015). Conversely, a long-term study may show if the parasite population's genetic diversity will instead eventually diminish due to the small population size of their hosts (Hazlitt *et al*., 2006).

The rarefaction analysis indicated that within the range of our analyses, sampling effort did not result in significant differences in OTU richness between population categories. However, a larger amount of sampling with true replication in a more invasive and extensive study across all three population categories would have approached the true OTU richness of

each population and may have provided enough statistical power to detect a difference if it exists. Such a study may also identify if a specific *Eimeria* OTU within an individual is maintained over time between the captive breeding facility and the release site. Furthermore, non-invasive collection of faecal samples prevented collection of data on host demographics (age and sex) which may potentially affect the *Eimeria* community structure (Hudson *et al*., 2002). A more extensive study that included tagging and trapping, would enable inclusion of demographic parameters or microsatellite marker analysis of the scats could be used to determine the individual's identity and sex (Hazlitt *et al*., 2006).

While no significant effect of population category was observed in this short-term study, continued large-scale studies over a longer period of time may produce different outcomes on *Eimeria* diversity or distribution. Site specificity of parasites is a possibility as dispersal of BTRW between populations is limited (Browning *et al*., 2001, Hazlitt *et al*., 2006). In the current study, a high overlap in OTU composition was detected between the geographically close sites (KV River and KV Creek). In comparison, hierarchal cluster analysis of OTU composition showed that Jenolan Caves was highly dissimilar from other sites. Jenolan Caves is ~120 km from Kangaroo Valley, ~350 km from Square Top, and separated from Nattai by the Blue Mountains. These findings suggest that the *Eimeria* diversity observed in BTRW may be due in part to biogeography. The majority of BTRW in NSW, including those at all the collection sites, belong to the central evolutionarily significant unit (ESU) (DECC, 2008, Hazlitt *et al*. 2014). Gene flow amongst the scattered populations of BTRW even within ESUs is highly restricted (Hazlitt et al., 2006) and the species is widely distributed from south-eastern Queensland (northern ESU) to Victoria (southern ESU). Future studies should examine the possible relationship between *Eimeria* diversity and biogeography, as well as the phylogeography of the host, through an extensive study of *Eimeria* community biodiversity within and between the three different BTRW ESUs.

This study revealed that the genetic diversity, richness, composition and relative prevalence of *Eimeria* OTUs was equally high in captive bred / supplemented animals compared to their wild counter-parts, suggesting that the biodiversity of the *Eimeria* communities was maintained throughout the conservation process. Furthermore, the *Eimeria* species from BTRW have not been previously sequenced and thus a future study using samples with a single OTU can compare both morphology of the oocysts to identify species and sequencing of a larger fragment of the 18S rRNA locus (Zhao & Duszynski 2001), which may aid to create more sensitive primers for BTRW-specific *Eimeria* species for future studies. In addition, this study revealed a high prevalence of *Eimeria* and no significant change in infection intensity between population categories. The maintenance of the biodiversity of a host specific parasite as an integral part of the parasite community within the host is a positive outcome for conservation management. However, we only examined the community structure of a single parasite genus yet hosts are likely to be infected with diverse parasite taxa (Rigaud *et al*., 2010, Rynkiewicz *et al*., 2015). As parasite communities form complex structures linked within and between parasite species, families and taxa (Ezenwa & Jolles, 2011, Knowles *et al*., 2013, Pedersen & Antonovics, 2013), variations in the number and genetic structure of one parasite species may have a greater effect on the parasite community as a whole and on the health of the host. Detrimental effects on parasite ecology and host health may be further compounded by adverse events such as habitat disruption (Barker *et al*., 1972). Therefore, we support further study into larger-scale parasite community analyses and management within the frame of conservation management strategies, to aid in the success of conserving biodiversity.

6.6 Acknowledgements

This research was funded by the Australian Research Council, Office of Environment and Heritage and the Australian Museum through the ARC Linkage program issued by Macquarie

University, Project ID: LP110200569. The animal ethics approval numbers are AEC 050207/02 and AEC 080728/01. The authors would like to thank Deborah Ashworth, Todd Soderquist, Celia Thomson, Melinda Norton and Juliet Dingle from Office of Environment and Heritage for collecting the BTRW faecal samples. We also thank David Nipperess from Macquarie University for his advice on statistical analyses. The authors declare that they have no conflict of interest.

6.7 Supplementary Material

Supplementary Table 6.1: Details of the faecal samples collected from brush-tailed rock-wallabies (*Petrogale penicillata*), including confirmation of *Eimeria* presence using microscopy or PCR/Post-NGS analysis. ND denotes 'no data' as these samples were not used for the zinc sulphate flotation count. KV denotes ‘Kangaroo Valley’.

Sample	Site	Population Category	Season	Year	*Eimeria* oocysts/g	Microscopy positive	18S rRNA PCR positive	*Eimeria* OTU Match
252	Waterfall Springs	Captive bred	Autumn	2010	4171	Yes	Yes	Yes
256	Waterfall Springs	Captive bred	Autumn	2010	21248	Yes	No	No
258	Waterfall Springs	Captive bred	Autumn	2010	2491	Yes	No	No
259	Waterfall Springs	Captive bred	Autumn	2010	471	Yes	No	No
491	Waterfall Springs	Captive bred	Winter	2010	68191	Yes	Yes	Yes
492	Waterfall Springs	Captive bred	Winter	2010	55501	Yes	Yes	Yes
494	Waterfall Springs	Captive bred	Winter	2010	20093	Yes	Yes	Yes
495	Waterfall Springs	Captive bred	Winter	2010	20216	Yes	Yes	Yes
543	Taronga Zoo	Captive bred	Winter	2011	ND	Yes	No	No
546	Taronga Zoo	Captive bred	Winter	2011	1412	Yes	No	No
547	Taronga Zoo	Captive bred	Winter	2011	19114	Yes	No	No
594	Square Top	Supplemented	Summer	2011	546	Yes	No	No
595	Square Top	Supplemented	Summer	2011	5141	Yes	No	No
602	Square Top	Supplemented	Summer	2011	4623	Yes	No	No
612	Waterfall Springs	Captive bred	Autumn	2012	7602	Yes	No	No
616	Waterfall Springs	Captive bred	Autumn	2012	9935	Yes	No	No
619	Waterfall Springs	Captive bred	Autumn	2012	5503	Yes	No	No
622	Waterfall Springs	Captive bred	Autumn	2012	3312	Yes	No	No
624	Waterfall Springs	Captive bred	Autumn	2012	11081	Yes	No	No
625	Waterfall Springs	Captive bred	Autumn	2012	6153	Yes	No	No
632	KV Mountain	Wild	Autumn	2012	103	Yes	Yes	Yes
633	KV Mountain	Wild	Autumn	2012	2529	Yes	Yes	Yes
644	KV River	Supplemented	Autumn	2012	2412	Yes	Yes	Yes
646	KV River	Supplemented	Autumn	2012	21210	Yes	No	No
647	KV River	Supplemented	Autumn	2012	56694	Yes	No	No
650	KV River	Supplemented	Autumn	2012	188820	Yes	Yes	Yes
652	KV River	Supplemented	Autumn	2012	74691	Yes	Yes	Yes
653	KV River	Supplemented	Autumn	2012	2183	Yes	Yes	Yes
654	KV River	Supplemented	Autumn	2012	10445	Yes	Yes	Yes

Supplementary Table 6.1 Continued

Sample	Site	Population Category	Season	Year	*Eimeria* oocysts/g	Microscopy positive	18S rRNA PCR positive	*Eimeria* OTU Match
656	KV River	Supplemented	Autumn	2012	10939	Yes	No	No
657	KV River	Supplemented	Autumn	2012	79658	Yes	No	No
666	KV Mountain	Wild	Autumn	2012	6237	Yes	Yes	Yes
667	KV Mountain	Wild	Autumn	2012	26653	Yes	No	No
675	KV Creek	Supplemented	Winter	2012	ND	Yes	Yes	Yes
678	KV River	Supplemented	Winter	2012	12386	Yes	No	No
681	KV River	Supplemented	Winter	2012	16904	Yes	Yes	Yes
682	KV River	Supplemented	Winter	2012	3176	Yes	Yes	Yes
686	KV Mountain	Wild	Winter	2012	16008	Yes	Yes	Yes
687	KV Mountain	Wild	Winter	2012	8253	Yes	No	No
688	KV Mountain	Wild	Winter	2012	7663	Yes	No	No
689	KV Mountain	Wild	Winter	2012	4434	Yes	No	No
690	KV Mountain	Wild	Winter	2012	12714	Yes	No	No
691	KV Mountain	Wild	Winter	2012	2935	Yes	No	No
693	KV Mountain	Wild	Winter	2012	8921	Yes	No	No
694	KV Mountain	Wild	Winter	2012	16770	Yes	No	No
695	KV Mountain	Wild	Winter	2012	20414	Yes	No	No
736	Square Top	Supplemented	Autumn	2009	ND	Yes	Yes	Yes
739	Square Top	Supplemented	Autumn	2009	ND	Yes	No	No
740	Square Top	Supplemented	Autumn	2009	ND	No	No	No
744	Square Top	Supplemented	Autumn	2009	ND	Yes	No	No
746	Square Top	Supplemented	Autumn	2009	ND	Yes	Yes	Yes
754	Square Top	Supplemented	Autumn	2009	ND	Yes	No	No
755	Square Top	Supplemented	Autumn	2009	ND	No	No	No
756	Square Top	Supplemented	Autumn	2009	ND	Yes	Yes	Yes
759	Square Top	Supplemented	Autumn	2009	ND	Yes	Yes	Yes
761	Square Top	Supplemented	Autumn	2009	ND	Yes	Yes	Yes
762	Jenolan Caves	Supplemented	Spring	2010	1030	Yes	No	No
763	Jenolan Caves	Supplemented	Spring	2009	24313	Yes	Yes	Yes
764	Jenolan Caves	Supplemented	Winter	2010	10869	Yes	Yes	Yes
767	Jenolan Caves	Supplemented	Spring	2010	1122	Yes	Yes	Yes
768	Jenolan Caves	Supplemented	Spring	2010	24546	Yes	Yes	Yes
772	Square Top	Supplemented	Summer	2011	ND	Yes	Yes	No
783	Square Top	Supplemented	Autumn	2011	ND	Yes	No	No
797	Square Top	Supplemented	Autumn	2011	ND	Yes	Yes	No
816	Jenolan Caves	Supplemented	Autumn	2011	232	Yes	Yes	Yes
819	Jenolan Caves	Supplemented	Autumn	2011	1528	Yes	No	No
820	Jenolan Caves	Supplemented	Autumn	2011	641	Yes	No	No

Supplementary Table 6.1 Continued

Sample	Site	Population Category	Season	Year	*Eimeria* oocysts/g	Microscopy positive	18S rRNA PCR positive	*Eimeria* OTU Match
822	Jenolan Caves	Supplemented	Autumn	2011	441	Yes	No	No
823	Jenolan Caves	Supplemented	Autumn	2011	166	Yes	No	No
824	Jenolan Caves	Supplemented	Autumn	2011	1594	Yes	No	No
833	KV River	Supplemented	Autumn	2010	6224	Yes	No	No
837	KV River	Supplemented	Summer	2010	3302	Yes	Yes	Yes
875	KV Mountain	Wild	Autumn	2010	6549	Yes	No	No
907	Nattai	Wild	Summer	2013	ND	Yes	Yes	Yes
909	Nattai	Wild	Summer	2013	ND	Yes	Yes	Yes
937	KV River	Supplemented	Autumn	2013	14196	Yes	Yes	Yes
941	KV River	Supplemented	Autumn	2013	1403	Yes	No	No
942	KV River	Supplemented	Autumn	2013	1364	Yes	No	No
947	KV Creek	Supplemented	Autumn	2013	ND	Yes	No	No
948	KV Creek	Supplemented	Autumn	2013	ND	Yes	Yes	Yes
951	KV Creek	Supplemented	Autumn	2013	10962	Yes	Yes	Yes
952	KV Creek	Supplemented	Autumn	2013	24393	Yes	Yes	Yes
961	KV River	Supplemented	Autumn	2013	20272	Yes	No	No
966	KV River	Supplemented	Autumn	2013	1261	Yes	Yes	Yes
969	KV River	Supplemented	Autumn	2013	4207	Yes	Yes	Yes
970	KV River	Supplemented	Autumn	2013	4390	Yes	Yes	Yes
972	KV River	Supplemented	Autumn	2013	10234	Yes	No	No
975	KV River	Supplemented	Autumn	2013	12480	Yes	No	No
976	KV River	Supplemented	Autumn	2013	461	Yes	Yes	No
982	Nattai	Wild	Winter	2013	48751	Yes	Yes	Yes
997	Square Top	Supplemented	Winter	2013	ND	No	No	No
1013	Square Top	Supplemented	Winter	2013	ND	No	No	No
1014	Square Top	Supplemented	Winter	2013	ND	No	No	No
1019	Square Top	Supplemented	Winter	2013	ND	No	No	No
1021	Square Top	Supplemented	Winter	2013	ND	No	No	No
1022	KV River	Supplemented	Autumn	2014	ND	Yes	Yes	Yes
1023	KV River	Supplemented	Autumn	2014	ND	No	No	No
1024	KV River	Supplemented	Autumn	2014	ND	Yes	Yes	Yes
1025	KV River	Supplemented	Autumn	2014	ND	Yes	No	No
1027	KV River	Supplemented	Autumn	2014	ND	Yes	Yes	Yes
1032	KV River	Supplemented	Autumn	2014	ND	Yes	Yes	Yes
1033	KV River	Supplemented	Autumn	2014	ND	Yes	Yes	Yes
1040	KV Creek	Supplemented	Summer	2014	ND	Yes	Yes	Yes
1041	KV Creek	Supplemented	Summer	2014	ND	Yes	Yes	Yes
1042	KV Creek	Supplemented	Summer	2014	ND	Yes	Yes	Yes

Supplementary Table 6.1 Continued

Sample	Site	Population Category	Season	Year	*Eimeria* oocysts/g	Microscopy positive	18S rRNA PCR positive	*Eimeria* OTU Match
1043	KV Creek	Supplemented	Summer	2014	ND	Yes	Yes	Yes
1044	KV Creek	Supplemented	Summer	2014	ND	Yes	No	No
1045	KV enclosure	Captive bred	Spring	2014	ND	Yes	Yes	Yes
1046	KV River	Supplemented	Winter	2014	ND	Yes	Yes	No
1047	KV River	Supplemented	Spring	2014	ND	No	No	No
1048	KV enclosure	Captive bred	Spring	2014	ND	Yes	Yes	Yes
1049	KV River	Supplemented	Winter	2014	ND	Yes	Yes	No
1050	KV enclosure	Captive bred	Spring	2014	ND	Yes	Yes	Yes
1051	KV Mountain	Wild	Winter	2014	ND	Yes	Yes	Yes
1052	KV enclosure	Captive bred	Spring	2014	ND	Yes	Yes	Yes
1053	KV Mountain	Wild	Winter	2014	ND	Yes	No	No
1054	KV enclosure	Captive bred	Spring	2014	ND	Yes	Yes	Yes

Supplementary Table 6.2: Background of the brush-tailed rock-wallabies (*Petrogale penicillata*) populations used in this study. KV denotes 'Kangaroo Valley'.

Site	Population Category	History
Jenolan Caves	Supplemented	Wild population supplemented with wild caught individuals from several sites in NSW and captive individuals from Waterfall Springs
KV Mountain	Wild	Undisturbed wild population
KV River	Supplemented	Wild population supplemented with wild caught individuals from Jenolan Caves and captive individuals from Waterfall Springs
KV Creek (Released)	Supplemented	Wild population supplemented with captive individuals from Waterfall Springs
KV Creek (Enclosure)	Captive bred	Captive individuals from Waterfall Springs
Nattai	Wild	Undisturbed wild population
Square Top	Supplemented	Wild population supplemented with captive individuals from Waterfall Springs
Taronga Zoo	Captive bred	Captive population sourced from introduced population from Kawau Island, New Zealand
Waterfall Springs	Captive bred	Captive population sourced from wild caught individuals from populations throughout central NSW and the introduced population in Kawau Island, New Zealand

Supplementary Table 6.3: All generated operational taxonomic units (OTUs) with their closest match in GenBank.

OTUs	Matched identity	Identity match %	Query cover %	Accepted as *Eimeria*
OTU 001	*Eimeria trichosuri* (FJ829320)	97	100	Yes
OTU 002	*Eimeria trichosuri* (FJ829320)	97	100	Yes
OTU 003	*Eimeria trichosuri* (FJ829320)	99	100	Yes
OTU 004	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	98	100	Yes
OTU 005	Uncultured stramenopile (GU823483)	99	100	No
OTU 006	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	96	100	Yes
OTU 007	*Cheilanthes tomentosa* (HQ157252) (fern)	99	100	No
OTU 008	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	90	100	Yes
OTU 009	*Eimeria trichosuri* (FJ829320)	90	100	Yes
OTU 010	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	96	100	Yes
OTU 011	*Cheilanthes tomentosa* (HQ157252) (fern)	99	100	No
OTU 012	Uncultured stramenopile (GU823483)	99	100	No
OTU 013	*Paraschneideria metamorphosa* (FJ459755) (apicomplexan)	91	100	No
OTU 014	*Cheilanthes tomentosa* (HQ157252) (fern)	99	100	No
OTU 015	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	89	100	Yes
OTU 016	*Cheilanthes tomentosa* (HQ157252) (fern)	99	100	No
OTU 017	*Cheilanthes tomentosa* (HQ157252) (fern)	99	100	No
OTU 018	*Eimeria innocua* (HG793045)	94	100	Yes
OTU 019	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	92	100	Yes
OTU 020	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	92	100	Yes
OTU 021	*Eimeria quokka* (KF225636)	99	**75**	No*
OTU 022	*Eimeria trichosuri* (FJ829320)	95	100	Yes
OTU 023	*Eimeria maxima* (FJ236333)	92	100	Yes
OTU 024	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	89	100	Yes
OTU 025	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	90	100	Yes

Supplementary Table 6.3 Continued

OTUs	Matched identity	Identity match %	Query cover %	Accepted as *Eimeria*
OTU 026	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	94	100	Yes
OTU 027	Uncultured stramenopile (FJ410588)	89	100	No
OTU 028	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	95	**76**	No*
OTU 029	Uncultured stramenopile (FJ410588)	90	100	No
OTU 030	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	95	100	Yes
OTU 031	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	93	100	Yes
OTU 032	Uncultured stramenopile (GU823483)	98	100	No
OTU 033	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	92	**75**	No*
OTU 034	Uncultured stramenopile (FJ410588)	90	100	No
OTU 035	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	89	100	Yes
OTU 036	*Cheilanthes tomentosa* (HQ157252) (fern)	98	100	No
OTU 037	Uncultured stramenopile (GU823483)	88	100	No
OTU 038	*Eimeria trichosuri* (FJ829320)	90	100	Yes
OTU 039	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	86	100	Yes
OTU 040	*Sartidia dewinteri* (HG965134) (grass)	97	100	No
OTU 042	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	88	100	Yes
OTU 043	Uncultured eukaryote (KF650040)	100	33	No
OTU 044	*Chlorella* sp. (KC492078) (algae)	93	100	No
OTU 045	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	90	100	Yes
OTU 046	*Eimeria quokka* (KF225636)	97	**63**	No*
OTU 047	*Proleptomonas faecicola* (GQ377682) (soil flagellate)	93	100	No
OTU 048	*Cryptococcus* sp. (KT279435) (fungus/yeast)	100	100	No
OTU 049	*Paraschneideria metamorphosa* (FJ459755) (apicomplexan)	89	100	No
OTU 050	*Chlorella* sp. (KC492078) (algae)	99	100	No
OTU 051	*Gloeodinium montanum* (EF058238) (dinoflagellate)	94	100	No
OTU 052	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	94	100	Yes

Supplementary Table 6.3 Continued

OTUs	Matched identity	Identity match %	Query cover %	Accepted as *Eimeria*
OTU 053	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	97	100	Yes
OTU 054	*Oryza sativa* (AP014965) (plant)	99	100	No
OTU 055	*Eimeria dispersa* (HG793041)	99	100	Yes
OTU 057	*Eimeria* sp. WGK2534 (from a macropod) (JF419344)	89	100	Yes
OTU 058	*Pholiota squarrosa* (DQ465337) (fungus)	99	100	No

*Query covers less than 100% may indicate an erroneous contig assembly and thus was treated as suspicious and left out of further analysis.

Supplementary Table 6.4: Summary of the mean, standard deviation and median (based on the Kruskal-Wallis test) of the oocysts per gram faeces count for each site.

Site	Mean	Standard Deviation	Median
Jenolan Caves	6724.50	9433.98	1325
KV Creek	8075.00	8266.27	6881
KV Mountain	9478.13	7585.55	7663
KV River	23304.67	41630.06	10340
Nattai	12944.25	23893.58	1513
Square Top	3436.67	2516.75	4623
Taronga Zoo	5530.75	9076.40	1438
Waterfall Springs	13552.94	18925.00	5828

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7 General Discussion

Conservation programs aim to abate the global decline of biodiversity (Meffe *et al*., 2006), yet an integral part of the biome, parasite communities, are often overlooked or only viewed from the perspective of disease management and not relative to conservation biology (Cranfield *et al*., 1994, Pizzi, 2009, Thompson *et al*., 2010a, Horan & Melstrom, 2011). Threatened hosts may have highly host specific parasites, which are at risk of co-extinction with the decline or extinction of their hosts (Gompper & Williams, 1998, Koh *et al*., 2004a, Koh *et al*., 2004b, Altizer *et al*., 2007, Dunn *et al*., 2009). As parasites aid development of the host immune system and are important drivers of biodiversity through coevolution, the maintenance of host specific parasites should have greater focus in conservation biology (Sousa, 1991, Nieberding *et al*., 2008, Paterson & Piertney, 2011). However, conservation management strategies may act to potentially lower the biodiversity of host specific parasites (Moir *et al*., 2012) and introduce novel, invasive parasite species to naive host populations (Cranfield *et al*., 1994). Yet, much is still unknown regarding the impact of conservation management on the structure and composition of parasite communities in threatened animals (Gompper & Williams, 1998, Pizzi, 2009, Gómez & Nichols, 2013).

In this study, I examined whether an ongoing conservation program had an impact on the parasite communities of a threatened marsupial, the brush-tailed rock-wallaby (BTRW, *Petrogale penicillata*). Firstly, I examined the parasite community of BTRWs in a broader context through examination of the published literature and the creation of a database of all parasites recorded to date in *Petrogale* (objective 1, Chapter 2). The parasite communities of the 17 species of rock-wallabies were found to be highly diverse, with 157 parasite species described to date, and highly host specific, showing correlations with both host phylogeny and biogeography. I then focused on the impact of a current conservation program for the BTRW on the generalist protozoan parasite species *Cryptosporidium* and *Giardia duodenalis*

through screening and identification of these parasites using polymerase chain reaction (PCR) (objectives 2 and 3, Chapters 3 and 4). The results showed that conservation management did not impact the prevalence and species / assemblage of these parasites. A different methodology was adopted to examine the complex genetic structure of *Eimeria* communities in BTRWs compared to *Cryptosporidium* and *G. duodenalis*. Using next generation sequencing (NGS), I developed methodology that was highly sensitive and accurate (objective 4, Chapter 5). This data analysis method was then combined with *Eimeria* infection intensity and prevalence through microscopy to examine the impact of conservation management on the *Eimeria* communities of BTRW (objective 5, Chapter 6). *Eimeria* community biodiversity was maintained across the conservation management program compared to wild populations and no significant impact was detected of conservation management on *Eimeria* infection intensity or prevalence.

7.1 Prevalence of host specific and broad host range parasites in BTRW that are part of a conservation program

My results showed that broad host range parasite species / assemblages had overall low prevalence in BTRWs and that conservation management had no detectable impact on their prevalence among BTRW population categories. The prevalence of *Cryptosporidium* and *Giardia* was low, similar to that reported previously in other marsupials, both wild and captive (Hill *et al*., 2008, McCarthy *et al*., 2008, Thompson *et al*., 2008, Thompson *et al*., 2010b, Ng *et al*., 2011). Conversely, the highly host specific parasite *Eimeria* was almost ubiquitous and did not differ significantly amongst BTRW population categories. The prevalence of *Eimeria* was equally high in other *Petrogale* (Barker *et al*., 1988b, O'Callaghan *et al*., 1998), macropodoid (Barker *et al*., 1988a, 1989, Hill *et al*., 2012, Austen *et al*., 2014) and marsupial species (Barker *et al*., 1979, Bennett *et al*., 2006). While coccidiosis in marsupials has been observed in rare cases (Barker *et al*., 1972, Munday, 1988, Hum *et al*.,

1991), the ubiquitous presence of host specific *Eimeria* species in marsupials may suggest low pathogenicity in these hosts.

The pathogenicity of *Eimeria* is often correlated with infection intensity (Hum *et al*., 1991, Daugschies & Najdrowski, 2005). Infection intensity is correlated with host population density (Winternitz *et al*., 2012) and is affected by the environment of the host as *Eimeria* spreads through the faecal-oral route (Daugschies & Najdrowski, 2005). As population density and environmental factors vary between captive breeding facilities and free range habitats, I aimed to investigate the effect of conservation management on infection intensity. While no significant effect between population categories was observed, there was a greater variation in oocyst shedding in populations supplemented with captive bred individuals. Longitudinal data collection of infection intensities of *Eimeria* in the population may resolve the large variation observed in supplemented populations. This data collection can indicate whether infection intensities become more stable over time in supplemented population or if the observed variation was driven by differences between individual BTRWs.

The full impact of conservation management on parasite community structures of threatened animals depends on the management practices involved. Captive breeding and translocation as management strategies have been discussed in this thesis, but in some cases, anti-parasite drugs such as anthelmintics are applied in zoos or as part of a captive breeding and release program (Arias *et al*., 2013, Robertson *et al*., 2015). While the application of drugs was not part of the BTRW conservation program, the use of targeted anti-parasite drugs in conservation programs may have an effect on parasite community structures (Pedersen & Antonovics, 2013). Thus, the role of drug use within conservation practices needs to be considered for management of threatened animals in general. Within any animal host, parasite taxa form complex communities that interact with each other (Rynkiewicz *et al*., 2015). Therefore, any impact on the infection intensity of one species or taxa can have an

effect on the other parasites in these communities (Knowles *et al*., 2013). For example, nematode infection intensity in captive BTRWs has been observed to be significantly lower than in wild individuals (Lott *et al*., 2012), which is opposite to the results of *Eimeria* infection intensity observed in the current study. A negative relationship has previously been observed between helminth burden and *Eimeria* infection intensity when anthelmintics were administered to the hosts (Knowles *et al*., 2013). It was proposed that the imbalance between parasite taxa may be due to anthelmintics increasing the infection intensity of non-target parasites (Pedersen & Antonovics, 2013). Furthermore, in captive systems and in the husbandry of domestic animals, the use of anthelmintics is of great concern towards parasites developing resistance against those drugs (Kenyon *et al*., 2009). Within a captive setting such as a zoo or a captive breeding facility, the type of housing and the density of animals within an enclosure can also have an effect on the parasite communities. *Cryptosporidium* prevalence and infection intensity in animals in a zoo, for example, was observed to be positively correlated with host density and how enclosed the housing was, with a lower parasite burden observed in large, open enclosures (Gracenea *et al*., 2002). Greater parasite prevalence and burden of protozoa and helminths have also observed in higher temperatures and humidity in both wild and captive hosts (Gracenea *et al*., 2002, Turner & Getz, 2010, Lott *et al*., 2014).

Based on the effect of seasonality and type of housing on parasite assemblages, it is recommended that captive bred individuals of threatened species intended for release are kept in large, open enclosures with as low a population density as possible, and that they are observed for parasite burdens during warmer and humid seasons, the impact of which on parasite burden will vary from country to country (Casemore, 1990). Little is known about the variation of parasite assemblages of BTRW in captivity and how they vary depending on seasonality, population density and type of housing. A long-term study of the seasonality and

transmission of parasite assemblages in captive BTRW will aid conservationists in developing policies to manage parasites through housing management for example. Such an investigation can be applied more broadly to parasite communities of other endangered species part of conservation management that aims to release individuals into depleted populations. With a greater understanding of parasite communities, more targeted management practices can be implemented that can prevent the loss of host specific parasites (Gompper & Williams, 1998, Gómez & Nichols, 2013) or the development of resistance against anti-parasitic drugs for pathogenic parasites (Kenyon *et al*., 2009).

7.2 Biodiversity of host specific and broad host range parasites in BTRW part of a conservation program

The biodiversity of host specific parasites (*Eimeria* and *C. fayeri*) appeared to be maintained across all BTRW population categories, as no significant difference was detected. However, there was a higher overlap in similarity of genotypes/operational taxonomic units (OTUs) for supplemented/captive bred isolates compared to wild isolates. The overlap observed for highly host specific parasite genotypes (*Eimeria* OTUs and *C. fayeri* genotypes at the *gp60* locus) between supplemented and captive bred populations may be due to the isolation of the captive bred animal population in the captive breeding facility. For example, differences in the nematode community structure of captive kangaroos have been observed previously between different captive breeding facilities and zoos (Lott *et al*., 2015). Thus, to determine if the genotype difference between populations is due to isolation in captive breeding facilities, the protozoan parasites of BTRW in different captive breeding facilities could be examined, as well as be compared with wild populations.

Previous identifications of *Eimeria* in BTRW have recorded only three species (Barker *et al*., 1988b), but the NGS data suggests that the genetic diversity of *Eimeria* communities in

BTRWs may be much greater than morphology alone indicates. *Eimeria* is highly diverse at the 18S rRNA locus however (Ogedengbe *et al*., 2011), and thus I could not attribute a single species to each OTU. Therefore, to know whether an OTU generated from NGS really matches a single species or multiple genotypes or alleles of the 18S rRNA of *Eimeria*, the genetic data from samples with a single OTU can be combined with examination of the morphology through microscopy and thus a previously recorded species or novel *Eimeria* species can be identified. By matching genetic data with morphological identification, a genetic barcode can be identified for each species (Ogedengbe *et al*., 2011). Using this genetic barcode, future studies can utilise NGS to screen *Eimeria* communities in any host once a database for its host specific *Eimeria* species has been created.

Broad range host parasite species / assemblages were also present across all three BTRW population categories and thus, the introduction of these parasites was not solely due to conservation management. *Cryptosporidium meleagridis* has a very broad host range, infecting a large range of birds and mammals (Ryan *et al*., 2014). This species was observed in both a captive bred isolate and several wild isolates with different subtypes at the highly variable *gp60* locus observed between these populations, possibly due to the isolation of captive bred individuals. Similarly, the anthropozoonotic assemblages A and B of *G. duodenali*s (Feng & Xiao, 2011) were observed in all population categories. The species / assemblages of *Cryptosporidium* and *G. duodenalis* that are broad in their host range have the potential to be pathogenic in humans and non-human animals (Thompson, 2000, Appelbee *et al*., 2005, Xiao & Feng, 2008, Feng & Xiao, 2011, Bouzid *et al*., 2013), but currently, their pathogenicity in marsupials is unknown with no report of clinical symptoms to date (Thompson, 2000, Power, 2010). The observed presence of the anthropozoonotic assemblages of *G. duodenalis* and the broad host range species *C. meleagridis* in BTRWs may yet be of some concern if sub-clinical infections become pathogenic. Stressors such as

disturbance, habitat disruption, co-infection or a lowered immune system may alter effect of *Giardia* or *Cryptosporidium* and affect the health of the host (Xiao & Feng, 2008, Elwin *et al*., 2012). *Cryptosporidium* for instance, is known to have a synergistic pathogenicity with other pathogens such as HIV in humans (Gatei *et al*., 2002, Cama *et al*., 2006, Ryan *et al*., 2014). Conservation actions did not appear to introduce broad host range parasite assemblages / species in this case as they were not exclusive to captive bred or supplemented populations. The presence of these anthropozoonotic parasites in wild BTRWs warrants further investigation into their transmission routes into BTRWs and other wildlife. One avenue of investigation into the transmission routes could involve testing bird faecal samples for *C. meleagridis* in the area of infection and compare the *gp60* subtype between the birds and BTRW. Similarly, water sources in the BTRW habitat could be investigated for the assemblages of *G. duodenalis* found in the BTRW.

However, comparison of genetic diversity of *G. duodenalis* between BTRW population categories was made difficult by mixed identifications at multiple loci or failure to amplify at some of the loci, a common problem in genotyping *G. duodenalis* in wildlife (Traub *et al*., 2004, Cacciò *et al*., 2008, Thompson *et al*., 2008). One issue may be that specific primers and PCR protocols commonly used to identify *G. duodenalis* were initially designed for human and domestic animal isolates (Hopkins *et al*., 1997, Read *et al*., 2002, Lalle *et al*., 2005). Primer specificity issues may be addressed by examining genetic data of *G. duodenalis* in wildlife and develop primers more specific to isolates from target host groups such as marsupials. NGS can be employed for whole genome sequencing of *G. duodenalis* from wildlife samples after cyst purification (Hadfield *et al*., 2015, Hanevik *et al*., 2015). Subsequently, the *G. duodenalis* genome can be matched to known assemblages or sub-assemblages through multi-locus genotyping (Cacciò *et al*., 2008) at known marker loci such as β-giardin (Lalle *et al*., 2005) or *tpi* (Traub *et al*., 2004). If the different genetic markers

within the *G. duodenalis* genomes from wildlife samples all match the same sub-assemblage known from previous studies human or domestic animals, then we can extrapolate the presence of known sub-assemblages in potential mixed infections through NGS community analysis similar to the approach for *Eimeria*. However, if the whole genome analysis reveals conflicting sub-assemblage identifications at different loci, then a new system of assigning sub-assemblages to wildlife isolates will need to be developed. It is also possible that conflicting identifications from different markers may indicate novel or cryptic (sub)assemblages and thus further study is required of the genetic identification of *G. duodenalis* isolates in wildlife.

Mixed infections were recorded of the host-specific *Eimeria* with both the marsupial host-specific *C. fayeri* (in captive bred isolates only) and the broad host range *C. meleagridis* (in wild isolates only) (see Appendix, Table 8.1). For *Giardia*, assemblage B was identified in those samples confirmed to be infected with *Eimeria*. Previous studies in marsupials have mainly focused on screening for *Cryptosporidium* and *G. duodenalis* simultaneously and identified both parasites in marsupial species, but it was unclear in these studies whether these parasites co-infected the same host (McCarthy *et al*., 2008, Ng *et al*., 2011). A study in a South-American marsupial, the white-eared opossum (*Didelphis albiventris*), confirmed through microscopy of faecal samples that a mixed infection of all three parasites is possible (Zanette *et al*., 2008), but such mixed infections have not yet been confirmed in Australian marsupials. This study confirmed mixed infections between *Eimeria* and *Cryptosporidium*, and *Eimeria* and *G. duodenalis*, occur in wild and captive bred BTRW.

Following the observations of unique OTUs and genotypes of *Eimeria* and *Cryptosporidium* in captive bred individuals, I proposed that this uniqueness may be due to their isolation over several generations from wild populations. However, the observation of differences between populations may actually be due to biogeographical constraints on parasite communities

rather than an effect of conservation management. Further studies could compare the parasite communities of the wild population from which the captive animals are caught, as well as compare between different captive breeding or zoo facilities to strengthen the hypothesis of the effect of biogeography on parasite communities. This hypothesis was strengthened by the observation of a greater overlap in similarity of *Eimeria* OTU richness between supplemented sites that were geographically close, compared to a highly isolated supplemented site with a more unique community structure compared to other sites. When comparing the parasite communities of all *Petrogale* species, a similar observation was made that biogeography had a highly significant impact of the parasite community structure of these wallabies. The biogeographical constraints are more apparent for more host specific parasites such as *Eimeria* and *C. fayeri*, while less apparent for a broad host range parasite like *G. duodenalis*. Previous studies in a range of different animals concur that there is a strong link between biogeography and phylogeny of particularly highly host specific parasites (Thompson, 1999, Hoberg & Brooks, 2008, Nieberding *et al*., 2008, Lott *et al*., 2012, Poulin *et al*., 2012, Froeschke & von der Heyden, 2014, Lott *et al*., 2015). Genetic analysis of the parasite communities in supplemented populations over a larger period of time may indicate if the genetic structure of parasite communities in these populations becomes more homogenous over time as captive bred animals come in contact with their wild counterparts.

7.3 Impact of conservation management on the enteric protozoan parasites of BTRW and future directions

In conclusion, there was no significant impact detected on the protozoan parasite communities of BTRWs that were part of this management program. Novel or broad host range species / assemblages were not observed more frequently in captive bred populations compared to wild populations. This study revealed a high host specificity of parasite communities in *Petrogale* as a whole and in BTRW in particular. The host specific genus

Eimeria and marsupial specific species *C. fayeri* were observed across all BTRW population categories, suggesting that host specific parasites were maintained in this conservation program. These findings and the review of previous literature suggest that variations observed in genetic diversity of these protozoan parasites in BTRW may be linked to biogeography rather than an impact by conservation management.

The conservation status of the host and its parasites may change over time however as external factors such as continuing human expansion, habitat fragmentation and future climate change may have a deeper impact on the parasite communities of threatened species than currently observed in this study (Holmes, 1996, McCallum & Dobson, 2002, Krauss *et al*., 2010, Minteer & Collins, 2010, Mawdsley, 2011). The negative impact of these external factors to the conservation of threatened species may be the loss of host specific parasites (Dobson *et al*., 2008, Moir *et al*., 2012) or exacerbation of stress factors on the host allowing infection of introduced, broad host range and potentially pathogenic species (Moberg, 1985, Cranfield *et al*., 1994). Host specific parasite communities are threatened with coextinction if their host populations become extinct, which means that the extinction of a single host species incurs a great loss to biodiversity and ecological systems (Koh *et al*., 2004a, Dobson *et al*., 2008, Dunn *et al*., 2009, Colwell *et al*., 2012). Therefore, conservation management programs need to adapt to conserve a complex community of organisms rather than focus on a single target species (Gompper & Williams, 1998). I propose larger-scale studies over longer time periods on the parasite communities of threatened animals, firstly to gain a more accurate estimate of their current biodiversity and secondly, to monitor their prevalence and biodiversity over time. The results of the current study however are encouraging for the current management practices of endangered species as this study could not detect a negative impact of the conservation strategy on the protozoan parasite communities of BTRW in New South Wales.

7.4 References

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8 Appendices

8.1 Appendix 1

Table 8.1: Faecal samples with mixed infections of *Eimeria* (confirmed through microscopy), *Cryptosporidium* (screening at 18S rRNA locus and multi-locus genotyping) and *Giardia duodenalis* (screening and ID across all three loci). KV denotes 'Kangaroo Valley'.

BW#	**Site**	**Population Category**	***Eimeria***	***Cryptosporidium* screening**	**Cryptosporidium ID**	***G. duodenalis* screening**	***G. duodenalis* assemblage**
612	Waterfall Springs	Captive bred	**Yes**	**Yes**	*C. fayeri* (same animal as 613)	No	N.A.
624	Waterfall Springs	Captive bred	**Yes**	**Yes**	*C. fayeri* (same animal as 613)	No	N.A.
625	Waterfall Springs	Captive bred	**Yes**	**Yes**	*C. fayeri* (same animal as 613)	No	N.A.
633	KV Mountain	Wild	**Yes**	**Yes**	18S only (*Cryptosporidium sp.*)	No	N.A.
666	KV Mountain	Wild	**Yes**	**Yes**	*C. meleagridis*	No	N.A.
667	KV Mountain	Wild	**Yes**	No	N.A.	**Yes**	BIV (*gdh* locus)
678	KV River	Supplemented	**Yes**	No	N.A.	**Yes**	B (18S rRNA locus)
689	KV Mountain	Wild	**Yes**	**Yes**	*C. meleagridis*	No	N.A.
691	KV Mountain	Wild	**Yes**	**Yes**	*C. meleagridis*	No	N.A.
694	KV Mountain	Wild	**Yes**	No	N.A.	**Yes**	BIV (*gdh* locus)
952	KV Creek	Supplemented	**Yes**	**Yes**	18S only (*C. parvum*)	No	N.A.
970	KV River	Supplemented	**Yes**	**Yes**	18S only (*C. parvum*)	No	N.A.