Prevalence, genetic diversity and potential clinical impact of blood-borne and enteric protozoan parasites in some Australian native mammals.

by

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A thesis submitted to Murdoch University to fulfil the requirements for the degree of Doctor of Philosophy in the discipline of Veterinary Sciences

School of Veterinary and Life Sciences

2017
Author’s declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Amanda Duarte Barbosa
Statement of contributors

The four experimental chapters presented in this thesis have been published as peer reviewed articles with multiple co-authors. The literature review is a modified version of a book chapter, currently in publication process, which has three co-authors. Amanda Duarte Barbosa was the first and corresponding author of these publications, primarily involved in conceiving ideas and project design, laboratory work, data analysis, and preparation and submission of manuscripts.

All publication co-authors have consented to their work being included in this thesis and have accepted this statement of contribution.
Abstract

Blood-borne and enteric protozoan parasites belonging to the genera *Trypanosoma*, *Babesia*, *Theileria*, *Cryptosporidium* and *Giardia* are responsible for severe animal and human illnesses worldwide. In addition, parasites of the genus *Hepatozoon* have been associated with animal morbidity and mortality. Despite recent research and improved knowledge of the taxonomy and distribution of native Australian protozoan parasites, still relatively little is known about their epidemiology, genetic diversity and pathogenicity. The overarching aim of this thesis was to determine the prevalence, molecular characterisation and potential clinical impact of protozoan pathogens of potential conservation and zoonotic importance in Australian native mammals.

A total of 465 blood samples, 167 faecal samples and 91 ticks were collected from mammals belonging to seven target species: common brush-tailed possums (*Trichosurus vulpecula*), northern brown bandicoots (*Isoodon macrourus*), northern quolls (*Dasyurus hallucatus*), brush-tailed rabbit-rats (*Conilurus penicillatus*), koalas, a little red flying fox (*Pteropus scapulatus*) and grey-headed flying foxes (*Pteropus poliocephalus*). The sampling was undertaken across four states/territories in Australia: the Northern Territory (NT), Queensland (Qld), New South Wales (NSW) and South Australia (SA). Molecular and morphological analyses were utilised to identify and characterise *Trypanosoma*, *Babesia*, *Theileria*, *Hepatozoon*, *Cryptosporidium* and *Giardia*. The potential clinical impact of the parasites identified was investigated by associating clinical, haematological and biochemical parameters, whenever available, with presence or absence of infection.

A molecular survey was conducted in the NT to investigate the prevalence, genetic diversity and potential pathogenicity of protozoan parasites in common brush-tailed possums, northern brown bandicoots, northern quolls, and brush-tailed rabbit-rats.
Overall, 22.5% (95% confidence interval (CI): 17.0-28.8%) of the animals tested were positive for haemoprotozoans by Polymerase Chain Reaction (PCR) targeting the 18S ribosomal RNA (rRNA) gene. *Trypanosoma vegrandid* and *T. noyesi* were found in 26.6% (95% CI: 18.7-35.7%) of the bandicoots and in 23.7% (95% CI: 11.4-40.2%) of the possums, respectively. *Babesia* spp. and *Hepatozoon* spp. were identified in bandicoots only, both at a prevalence of 5.3% (95% CI: 2.7-9.3%). *Hepatozoon* gamonots were detected using light microscopy in two out of 11 animals positive for this parasite by PCR.

Faecal samples were tested for *Cryptosporidium* spp. at the 18S rRNA locus, and for *Giardia* spp. at the glutamate dehydrogenase (gdh) and 18S rRNA loci. The total prevalence of intestinal protozoan parasites observed was relatively low (3%; 95% CI: 1.0-6.9%). No clear signs of major morbidity were observed in infected animals, however bandicoots positive for *Trypanosoma* exhibited a significantly lower packed cell volume (PCV) compared to negative bandicoots (p = 0.046).

The first report of *T. vegrandid* in koalas using morphology and sequence analysis of the 18S rRNA gene is also described. The prevalence of *T. vegrandid* in koalas was (13.6%; 95% CI: 5.2-27.4%). In addition, a novel next-generation sequencing (NGS)-based assay for *Trypanosoma*, developed during the present study, revealed that mixed infections with up to five trypanosome species in koalas were significantly more prevalent (27.4%; 95% CI: 21-35%) than single trypanosome infections (4.8%; 95% CI: 2-9%). Infections with *T. gilletti*, *T. irwini*, *T. copemani* and *T. vegrandid* were identified. Additionally, *T. noyesi* was detected for the first time in koalas, although at a low prevalence (0.6%; 95% CI: 0-3.3%), and a novel species (*Trypanosoma* sp. AB-2017) was identified at a prevalence of 4.8% (95% CI: 2.1-9.2%). Overall, a considerably higher proportion (79.7%) of the *Trypanosoma* sequences isolated from
koala blood were identified as *T. irwini*, suggesting this was the dominant species. The study also employed the NGS methodology to profile trypanosome communities within *Ixodes holocyclus* and *I. tasmani* ticks removed from koala hosts. Co-infections involving *T. gilletti*, *T. irwini*, *T. copemani*, *T. vegrandis* and *Trypanosoma* sp. AB-2017 were also detected in the ticks, with *T. gilletti* and *T. copemani* being the dominant species within the invertebrate hosts.

This thesis also characterised a novel trypanosome species in a little red flying fox with clinical signs of trypanosomiasis, using morphology and molecular analyses at the 18S rRNA and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) loci. Morphological comparison showed that trypomastigotes of the novel species were significantly different from those of *Trypanosoma pteropi* and *T. hipposideri*, two species previously described from Australian bats for which genetic information was unfortunately not available. Phylogenetic analysis revealed that the novel species was genetically distinct and clustered with other bat-derived trypanosome species within the *Trypanosoma cruzi* clade. The discovery of a new bat-derived trypanosome species in Australia prompted the screening of an additional 87 blood samples from grey-headed flying foxes, which were negative for *Trypanosoma* 18S rDNA.

In summary, this research provides new insights on the prevalence, spatial distribution, inter- and intra-specific genetic diversity and the potential negative effects of blood-borne and enteric protozoan parasites on the health of Australian mammals. Furthermore, the identification of trypanosome polyparasitism in koalas and two species of native ticks will inform future epidemiological conservation studies. The outcomes of this thesis may be used to inform wildlife management and zoonotic disease programs.
Acknowledgements

First and foremost, I would like to thank God for the health and capacity to undertake this research as well as for the opportunity to meet the people acknowledged below, who inspired and supported me throughout this journey.

I wish to express my deepest gratitude to my supervisors: Professor Una Ryan, Professor Peter Irwin, A/Professor Kristin Warren and Dr Andrea Paparini. Una, you have been not only a mentor but also a source of inspiration, role model and a pillar of support and encouragement. Thank you for sharing your exceptional knowledge and for believing in my potential and abilities. Peter, thank you so much for your invaluable guidance and for sharing your expertise. Kris, you have greatly contributed to the success of this research (particularly by assisting with the establishment of our collaborative network of wildlife experts in Australia), thank you. Andrea, I am truly grateful for your constructive comments and for teaching me important laboratory and bioinformatics skills.

I am thankful for the support given by all of my colleagues from the Vector and Water-borne Pathogens Research Group at Murdoch University. The insightful discussions, credible ideas and collaborations in a range of exciting research projects have been great contributors in my learning process and in the completion of this thesis. In particular, I thank Dr Charlotte Oskam for her enthusiasm and advice on early career research steps; Dr Jill Austen for her illuminating advice and our fun moments of hard work together; Alex Gofton for his valuable assistance with next-generation sequencing; Kamil Braima for his kind assistance with the thesis formatting; Alireza Zahedi and Dr Rongchang Young for providing helpful advice.
I acknowledge the support and assistance provided by our collaborators Dr Amber Gillett (Australia Zoo Wildlife Hospital - AZWH), Dr Karrie Rose (Taronga Zoo), Dr Timothy Portas (Tidbinbilla Reserve), Dr Wayne Boardman (University of Adelaide), Ms Cheyne Flanagan (Koala Hospital), Dr David McLelland (Zoos SA), Dr John Mackie (Vepalabs), Robin Stenner (AZWH), Dr Andrea Reiss (Zoo and Aquarium Association) and Dr Bethany Jackson (Murdoch University). I would also like to acknowledge the support extended by my office mates (Khalid, Kamil, Diana and Cindy) and staff at Murdoch University, particularly Janice Stigwood, Ken Chong, Frances Brigg, David Berryman, Gordon Thompson, Dale Banks, Dr Lisellotte Pannier, Dr Mark O’Dea and Dr Sam Abraham.

I would like to acknowledge the considerable financial support from CAPES Foundation, Ministry of Education of Brazil, and the Australian Society for Parasitology (ASP) for the student travel grant to present my work at the ASP annual conferences.

My acknowledgements would be incomplete without thanking the greatest source of my strength and inspiration, my family. I am profoundly grateful to my parents Mr Antonio Barbosa and Mrs Maximina Barbosa for having invested in my education, taught me strong ethical values and encouraged me to pursue my dream even though this meant living thousands of miles away. Mum and dad, this achievement would not have been possible without your help and unwavering love. Special thank you goes to my big brother Thiago for his continuous support and invaluable advice during these years and my whole life. Thank you also to my sister-in-law Luiza for her friendship and warm encouragement. In fact, I am grateful to Thi and Lu in particular for bringing Thomas and Livia to the world. These two little ones have filled my life with joy and never let things get dull or boring. I also thank Zecca and Lizbela, two special family members, for their unconditional love.
To the other members of our ‘Braussie family’ (Flavia, Carol, Marcio and Anna) thank you for literally being a family to me in Perth. Fla, thank you so much for the friendship and support particularly when I first arrived in Australia. I will never forget what you have done to encourage me to go ahead. I am also grateful to Fernanda and Tingting for the kind words and great company during my not very frequent leisure moments. Amanda, Juliana Lara, Andre, Marcelle, Juliana Faria, Clarice, Patricia, Thais and Carol, thank you for your true friendship. Despite the physical distance, we have managed to stay close and this has been of great importance to me during this journey.
Publications arising from this thesis

. Book chapter


. Journal publications


Conference oral presentations


**Conference poster presentations**


- **Media announcements**

Barbosa, A. Murdoch researcher helping to save Australian koalas, 2017 (Interview)

Murdoch University, WA


Barbosa, A. Blood-sucking ticks a new threat to health of koalas, 2017 (Interview)

Lismore echo, NSW


Barbosa, A. Parasites key to koala illness, 2017 (Interview)

The McIvor Times, Vic


- **Collaborations**

The author of this thesis also collaborated in a range of other research projects related to the subject of the present work. The collaboration resulted in the co-authorship of several manuscripts:


**A note on thesis layout**

This thesis consists of chapters that have been prepared as stand-alone manuscripts for publication in journals. To maintain contents and formatting consistency throughout the thesis, the chapters here presented differ slightly from the corresponding published manuscripts.

Chapter 1 (Literature review) is a modified version of a book chapter reviewed and approved by the editors. The book is currently in publication process.
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<td>μL</td>
<td>Microlitre</td>
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<td>ACT</td>
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<td>FF</td>
<td>Free flagellum measurement</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>gdh</td>
<td>Glutamate dehydrogenase gene</td>
</tr>
<tr>
<td>GGT</td>
<td>Gamma glutamyl transferase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Gamma interferon</td>
</tr>
<tr>
<td>Glob</td>
<td>Globulin</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est – in other words</td>
</tr>
<tr>
<td>k</td>
<td>Kinetoplast</td>
</tr>
<tr>
<td>KN</td>
<td>Kinetoplast to nucleus measurement</td>
</tr>
<tr>
<td>KoRV</td>
<td>Koala retrovirus</td>
</tr>
<tr>
<td>M</td>
<td>Molar concentration</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean cell haemoglobin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean cell haemoglobin content</td>
</tr>
<tr>
<td>MCV</td>
<td>Mean cell volume</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi Locus Sequence Typing</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>n</td>
<td>Number</td>
</tr>
<tr>
<td>NA</td>
<td>Nucleus to anterior measurement</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-generation sequencing</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Wales</td>
</tr>
<tr>
<td>NT</td>
<td>Northern Territory</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational Taxonomic Unit</td>
</tr>
<tr>
<td>p</td>
<td>Probability of an event due to chance alone</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>Packed cell volume</td>
</tr>
<tr>
<td>pers. comm.</td>
<td>Personal communication</td>
</tr>
<tr>
<td>PK</td>
<td>Posterior to the kinetoplast measurement</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomoles</td>
</tr>
<tr>
<td>PN</td>
<td>Posterior to nucleus measurement</td>
</tr>
<tr>
<td>Qld</td>
<td>Queensland</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>sp.</td>
<td>Unknown species (singular)</td>
</tr>
<tr>
<td>spp.</td>
<td>Several species</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>18S ribosomal RNA</td>
</tr>
<tr>
<td>SA</td>
<td>South Australia</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em> deoxyribonucleic acid polymerase</td>
</tr>
<tr>
<td>Tas.</td>
<td>Tasmania</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>TL</td>
<td>Total length</td>
</tr>
<tr>
<td>tpi</td>
<td>triose phosphate isomerase</td>
</tr>
<tr>
<td>TRCC</td>
<td>Total red cell count</td>
</tr>
<tr>
<td>TTP</td>
<td>Total plasma protein</td>
</tr>
<tr>
<td>TWCC</td>
<td>Total white cell count</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>Vic</td>
<td>Victoria</td>
</tr>
<tr>
<td>WA</td>
<td>Western Australia</td>
</tr>
</tbody>
</table>
Chapter 1  Literature Review: Blood-borne and enteric protozoan parasites of Australian native mammals

1.1 Preface

This chapter is a modified version of the book chapter:

1.2 Introduction

Compared to other continents, Australia has unique mammal fauna which have co-evolved in relative isolation since the complete separation of the continent from the Gondwana landmass ~ 40 million years ago (Long, 2017). Current estimates account for approximately 306 species of terrestrial and arboreal mammals that were extant in Australia at the time of European settlement in 1788 (McKenzie et al., 2007). This includes 162 marsupial species, 66 rodents, 76 bats and two monotremes (McKenzie et al., 2007; Van Dyck & Strahan, 2008).

For many years, the unique mammals of Australia have been under increased pressure posed by human encroachment. However, only more recently, after the extinction of many native species, concern over wildlife population declines have led to efforts to investigate and mitigate the causes for these events. In this context, increasing attention has been given to the potential clinical impact of protozoan parasites on Australian native mammals and also the role these animals may play as reservoirs of zoonotic pathogens.

Blood-borne protozoan parasites are unicellular eukaryotic organisms with sophisticated life-cycles alternating between development in the tissues and blood of a range of vertebrate hosts and the gut and tissues of various blood-feeding invertebrate vectors (O'Donoghue, 2017). Five groups of haemoprotozoan parasites are recognized (Lee et al., 2000), including trypanosomatids, haemogregarines and piroplasms (Fig. 1.1). Some species belonging to these groups are responsible for significant animal and human illnesses worldwide and appear to have the potential to adversely affect the health of Australian native fauna (Backhouse & Bolliger, 1957; Mackerras, 1959; Barker et al., 1978; Austen et al., 2009; McInnes et al., 2011a; Botero et al., 2013; Dawood et al., 2013; Thompson et al., 2014b; Donahoe et al., 2015).
Cryptosporidium and Giardia are ubiquitous protozoan parasites of the small intestine and stomach of vertebrates (Fletcher et al., 2012; Checkley et al., 2015). These parasites, transmitted by the faecal-oral route, are able to infect a broad range of hosts including humans, domestic and wild animals worldwide, causing asymptomatic or mild to severe gastrointestinal disease in their hosts (Monis & Thompson, 2003; Xiao, 2010; Ryan & Power, 2012; Ryan et al., 2014; Zahedi et al., 2016b). The impact of Cryptosporidium and Giardia on the health of wildlife is not known.

Australian ecosystems have been relatively under-explored for protozoan pathogens. However, investigations conducted to date have identified exotic and zoonotic pathogens in Australian native mammals (Wyatt et al., 2008; Thompson & Ash, 2016). In addition, several species and genotypes that seem unique to Australia have been recorded in these native hosts (Paparini et al., 2012b; Thompson et al., 2014a; Thompson & Ash, 2016; Cooper et al., 2017). Whilst protozoan parasites and wildlife, in general, seem to have co-evolved and developed subclinical host-parasite relationships, some usually asymptomatic agents can become pathogenic when the ecological balance is disrupted and stress factors emerge (Thompson et al., 2018).

Despite the growing diversity of recently described native Australian protozoan parasites, there is still relatively limited knowledge on their prevalence, genetic diversity, host-range, spatial distribution, vectors, pathogenicity and zoonotic potential. Furthermore, there has been very limited molecular surveillance of exotic protozoan pathogens in Australian wildlife, which is important both from a biosecurity and a One Health perspective. Further investigations into protozoan parasites and their potential vectors therefore constitute a current research priority in Australia, as these organisms could potentially be a neglected cause of parasitic diseases in wildlife and also in humans (Thompson & Thompson, 2015).
This chapter describes the current knowledge on the epidemiology and clinical impact of protozoan parasites of conservation and zoonotic importance documented in Australian mammals. This review will also note the diagnostic methodologies that have been applied to characterising and studying these organisms.

Figure 1.1. Key characteristics of trypanosomes, haemogregarines and piroplasms (encompassed in the present study) in the context of all five haematozoa assemblages (O’Donogue, 2017).

1.3 Trypanosomes

Trypanosomes are flagellated dixenous protozoan parasites which form asexual developmental stages (extracellular epimastigotes or promastigotes) in vertebrate hosts and invertebrate vectors. Whilst some trypanosome species can cause serious human diseases such as African and American trypanosomiasis (sleeping sickness and Chagas’ disease, respectively), the majority of wildlife trypanosomes have historically been considered benign in their vertebrate hosts (Hoare, 1972; Thompson et al., 2014a).
In terms of transmission dynamics, trypanosomes are divided in two groups: Salivaria and Stercoraria. The former undergo morphological and physiological transformation in the salivary glands of the vectors and are transmitted by inoculation during a blood meal; whereas the latter reside in the invertebrate vector’s gut and are transmitted by the faecal route, through contamination and penetration of bite wounds or mucosal membranes. Another possible route of transmission is via ingestion of an infected vector by the host (Hoare, 1972).

The basic morphology of bloodstream trypanastigotes is somewhat lanceolate and oval in transverse section; containing a kinetoplast (an extranuclear mass of DNA near the posterior end) and a single undulating flagellum at the anterior end (Fig. 1.2). Recent studies have demonstrated great morphological diversity within and between trypanosome species in Australia, with reported average lengths ranging from 8.3 μm to 38 μm, and average widths ranging from 1.3 μm to 15 μm (Austen et al., 2009; McInnes et al., 2009; Thompson et al., 2013; Cooper et al., 2017). *Trypanosoma vegrandis* recorded in Australian marsupials and bats, is believed to be the smallest trypanosome species formally described from mammals (8.3 μm in length and 1.3 μm in width) (Thompson et al., 2013).
Figure 1.2. *Trypanosoma copemani* isolated from the blood of a Gilbert’s potoroo (*Potorous gilbertii*) a) Light micrograph, scale bar represent 10 µm. b) Scanning electron micrograph of *T. copemani* from *in vitro* culture originally isolated from a Gilbert’s potoroo. Images courtesy of Dr Jill Austen.

Australian trypanosomes also exhibit high intra- and inter-species genetic diversity. Interestingly, phylogenetic studies have demonstrated that some indigenous species are more closely related to species outside Australia. For instance, *Trypanosoma noyesi* is genetically more similar to *T. cruzi* and bat-derived trypanosomes from South America and Africa, respectively than to other Australian native marsupial-derived trypanosomes, such as *T. irwini*, *T. copemani*, *T. gilletti* and *T. vegrandis* (Hamilton et al., 2004; Hamilton et al., 2012; Thompson et al., 2014a; Cooper et al., 2017). A recent study has hypothesised that bat trypanosomes may be precursors of terrestrial lineages of trypanosomes within the *T. cruzi* clade, including those from Australian marsupials (Hamilton et al., 2012).

To date, 68 native Australian mammal species have been screened for trypanosomes; including 46 of the 162 marsupial species (28%), 9 of the 66 rodent species (14%), 11 of the 76 bat species (14%) and both monotreme species (100%) (Thompson et al.,
2014a; Austen et al., 2015a). From these, 10 native *Trypanosoma* spp. have been taxonomically described and nine genotypes reported (Table 1.1).

**Table 1.1. Trypanosomes of native Australian mammals**

<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Host (s)</th>
<th>Origin/Distribution</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trypanosoma binneyi</em> 2,3,5,6,8,9,18</td>
<td>Platypus (<em>Ornithorhynchus anatinus</em>)</td>
<td>Tas.</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. irwini</em> 13-15</td>
<td>Koala (<em>Phascolarctos cinereus</em>)</td>
<td>NSW and Qld</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. gilletti</em> 14,15</td>
<td>Koala</td>
<td>NSW and Qld</td>
<td>Associated with lower PCV, lower body condition score, decreased survival in koalas with concurrent infections e.g., <em>Chlamydia</em> spp., KoRV</td>
</tr>
<tr>
<td><em>T. copemani</em> 10-12,14-17,19,21</td>
<td>Quokka (<em>Setonix brachyurus</em>)</td>
<td>NSW, Qld, WA</td>
<td>Inflammation, tissue degeneration, necrosis in woylies’ heart, skeletal muscle, oesophagus, tongue<em>17</em> Implicated in woylie population decline<em>19</em></td>
</tr>
<tr>
<td></td>
<td>Southern brown bandicoot (<em>Isoodon obesulus</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tiger quoll (or spot-tailed quoll) (<em>D. maculatus</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Woylie (or brush-tailed bettong) (<em>B. penicillata</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. vegrandis</em> 16,17,20</td>
<td>Southern brown bandicoot</td>
<td>NSW, Qld, WA</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Koala</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tammar wallaby (<em>Macropus eugenii</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Western grey kangaroo (<em>M. fuliginosus</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Western quoll (<em>D. geoffroii</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Woylie</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gould’s wattled bat (<em>Chalinolobus gouldii</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lesser long-eared bat (<em>Nyctophilus geoffroyi</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Black flying fox (<em>Pteropus alecto</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Little red flying fox (<em>P. scapulatus</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypanosome species</td>
<td>Host (s)</td>
<td>Origin/Distribution</td>
<td>Clinical significance</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------</td>
<td>---------------------</td>
<td>-----------------------</td>
</tr>
</tbody>
</table>
| *T. noyesi* 5,12,16,17,22 | - Eastern grey kangaroo (*M. giganteus*)  
- Common brush-tailed possum  
- Burrowing bettong (*B. lesueur*)  
- Woylie  
- Banded hare-wallaby (*Lagostrophus fasciatus*)  
- Swamp wallaby (*Wallabia bicolor*)  
- Bush rat (*Rattus fuscipes*) | Vic, WA | Unknown |
| *T. thylacis* *3* | Northern brown bandicoot | Qld | Unknown |
| *T. pteropi* *3* | Black flying-fox | Qld | Unknown |
| *T. hipposideri* *3* | Dusky leaf-nosed bat (*Hipposideros ater*) | Qld | Unknown |
| *Trypanosoma* sp. *23* | Little red flying fox | Qld | Anaemia, icterus, haemorrhage, acute haemoglobinuric nephrosis consistent with intravascular haemolysis reported in one individual *22* |
| *Trypanosoma* sp. isolate ABF *8* | Swamp wallaby | NSW | Unknown |
| *Trypanosoma* sp. *4,5* | Eastern barred bandicoot (*Perameles gunnii*) | Tas., Vic | Unknown |
| *Trypanosoma* sp. *12* | - Golden bandicoot (*I. auratus*)  
- Shark Bay mouse (*Pseudomys fieldii*) | WA | Unknown |
<p>| <em>Trypanosoma</em> sp. <em>5,9</em> | Brush-tailed rock-wallaby (<em>Petrogale penicillata</em>) | Vic | Unknown |
| <em>Trypanosoma</em> sp. <em>5,8,9</em> | Swamp wallaby | Vic | Unknown |
| <em>Trypanosoma</em> sp. <em>12</em> | Burrowing bettong | WA | Unknown |</p>
<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Host (s)</th>
<th>Origin/ Distribution</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trypanosoma</em> sp. 12</td>
<td>Dibbler (<em>Parantechinus apicalis</em>)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Trypanosoma</em> sp. 12</td>
<td>Pygmy planigale (<em>Planigale maculata</em>)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
| *T. lewisi* | - Water rat (*Hydromys chrysogaster*)  
- Bush rat (*Rattus fuscipes*)  
- Ash-grey mouse (*Pseudomys albocinereus*) | WA | Unknown |
| *T. cruzi* | Short-beaked echidna (*Tachyglossus aculeatus*) | N/A | Acute trypanosomiasis similar to South American trypanosomiasis or Chagas disease (fever, anorexia, enlarged lymph glands); death due to heart failure. |
| *T. evansi* | Agil wallaby (*M. agilis*) | N/A | Death or sickness characterised by anorexia, ataxia, weakness, anaemia |


*Species classification based on morphological characterisation only
*Exotic species introduced to Australia
# Animals experimentally infected with trypanosomes
N/A=Not available

**1.3.1 Epidemiology**

There is limited knowledge on the prevalence of trypanosomes in Australian mammals. Molecular studies have detected trypanosomes in the blood of up to 74% of koalas.
(Phascolarctos cinereus) and woylies (or brush-tailed bettongs) (Betongia penicillata) (McInnes et al., 2011a; Botero et al., 2013). A high prevalence has also been reported in platypuses (Ornithorhynchus anatinus) (86%) and various species of bats (82%) (Paparini et al., 2014; Austen et al., 2015a). Determining the true prevalence of trypanosomes in native mammals, especially rare species, is challenging given the relatively small sample sizes and restricted geographical distribution of most investigations conducted to date. Furthermore, prevalence data may vary between different studies, influenced by the techniques used for parasite detection and due to intermittent parasitaemia during the natural course of infection (Botero et al., 2013).

Studies to date have indicated that Australian trypanosomes appear to have a widespread geographic distribution (Thompson et al., 2014a). Additionally, there are many vertebrate populations across the Australian mainland and islands that have not yet been examined, meaning that current knowledge on the biogeography of Australian trypanosomes is far from complete.

Whilst there is no conclusive evidence on the identity of invertebrate vectors of trypanosomes in Australian native species, various ectoparasites including fleas, tabanid flies and several tick species (Ixodes spp., Haemaphysalis spp., Amblyomma triguttatum) have been reported as potential vectors (Mackerras, 1959; Noyes et al., 1999; Hamilton et al., 2005; Austen et al., 2011; Paparini et al., 2014; Botero et al., 2016b).

In contrast to the wealth of information on zoonotic trypanosomes, little is known about the life-cycle of Australian trypanosomes. A recent study has described promastigotes, sphaeromastigotes, amastigotes and new life-cycle stages of T. copemani within the blood stream of quokkas (Austen et al., 2015b). Interestingly, one of the newly reported
stages appeared to adhere to erythrocytes potentially causing haematological damage to the hosts (Austen et al., 2015b). The life-cycle of *T. copemani* G2 has recently been proposed (Fig. 1.3) (Botero et al., 2016a). To date, this is the only Australian genetic variant known to be able to infect cells.

The transmission dynamics of most Australian indigenous trypanosomes is not well elucidated. The recent detection of *T. copemani* intact trypomastigotes in the faeces of the tick *Ixodes australiensis* after 30 days of incubation suggested that transmission of this species occurs via the faecal-oral route (Austen et al., 2011).

![Figure 1.3. Proposed life cycle of *Trypanosoma copemani* G2 in the vertebrate host (Botero et al., 2016b).](image)

1.3.2 Pathogenesis and clinical significance of trypanosomiasis in Australian native mammals

Whilst subclinical trypanosome infections are common in wildlife, disease can result in morbidity and mortality, particularly in immunocompromised hosts. Haemolytic
anaemia is common and may be a consequence of the physical effects of the parasites (e.g. mechanical injury to erythrocytes and release of proteases) or due to extravascular haemolysis arising from antibody-mediated erythrocyte destruction (Clark et al., 2004; Mackie et al., 2017). In addition to the effects on erythrocytes, trypanosomes can invade host cells (e.g. *T. copemani*), causing tissue inflammation, reducing fitness of the host, and thereby potentially increasing susceptibility to predation (Botero et al., 2013).

Trypanosomes can also potentiate the effects of concurrent infections by compromising the immune system of their hosts (Khan & Lacey, 1986; Goossens et al., 1997; Carrera et al., 2009). Trypanosome-induced immunosuppression involves various mechanisms of depression of cellular and humoral immune responses, including quantitative, biochemical and functional changes of T cells, B cells and macrophages (Zuniga et al., 2000; Vincendeau & Bouteille, 2006).

In Australia, the awareness of the potential clinical significance of trypanosomes in Australian wildlife is increasing, especially as more mammals become endangered. Although most indigenous trypanosomes identified to date are thought to be non-pathogenic (Thompson et al., 2014a; Cooper et al., 2017), there are several documented cases (discussed below), suggesting that some of these species have the potential to adversely affect the health of their hosts.

Additionally, exotic trypanosome species may also have a negative impact upon the health of naïve mammalian hosts in Australia (Abbott, 2006; Thompson et al., 2014a). *Trypanosoma lewisi* is the only exotic trypanosome identified in Australian native mammals to date, and has been associated with the extinction of endemic rodents.
1.3.2.1 **Trypanosoma lewisi and extinction of endemic rats of Christmas Island**

Following the unintentional introduction of black rats (*Rattus rattus*) infected with *T. lewisi* and their flea vectors to Christmas Island in the early 1900s, two endemic rodent species, Maclear’s (*R. macleari*) and bulldog (*R. nativitatis*) rats became extinct. Reports at the time described Maclear’s rats being frequently found sick or moribund and heavily infected with trypanosomes and demonstrating pathological changes consistent with trypanosome infection at necropsy (Andrews, 1909). A century after their extinction, molecular analysis of ancient DNA suggested that native trypanosomes were absent from the endemic rodents on Christmas Island prior to introduction of the black rat (Wyatt et al., 2008), lending weight to the hypothesis that *T. lewisi* infection contributed to the extinctions.

1.3.2.2 **Trypanosoma spp. and koala morbidity**

The koala is an iconic Australian marsupial that is under threat of extinction across two thirds of its range, with population declines of over 50% being reported in some states (McAlpine et al., 2015). Of the factors contributing to koala population declines, infectious disease is a major cause of mortality (both in its own right and through predisposing animals to vehicle strike or dog attack), and infertility in the koala (Rhodes et al., 2011).

*Chlamydia* and koala retrovirus (KoRV) are known to be significant causes of infectious disease in koalas (Polkinghorne et al., 2013). Although trypanosome infections in koalas may be asymptomatic, clinical signs of trypanosomiasis have been reported, including poor body condition and regenerative anaemia due to extravascular haemolysis. Trypanosomes have been identified on blood films with a regenerative erythroid response evident in blood and bone marrow. Some cases progressed to developing neurological signs such as nystagmus, tremors and seizures; however, it is
unclear if these signs could be attributed to the trypanosomes. Histopathology revealed small organisms in the liver and central nervous system suggestive of trypanosome intracellular amastigotes, however their identity could not be confirmed. Other findings included lymphocytic/plasmacytic choroiditis in the animals with neurological signs, with putative trypanosomes in some of the choroid vessels (A Gillett pers. comm.).

A recent study provided further evidence that trypanosome infections may also impact koala health and survival and may be contributing to the decline of koala populations in eastern Australia (McInnes et al., 2011a). A significant association was reported between infection with *T. gilletti* and low PCV and body condition scores in koalas with signs of concurrent diseases (chlamydiosis, bone marrow dysplasia or KoRV-associated immune deficiency). This suggests that normally benign trypanosome infections may become pathogenic in koalas that are immunosuppressed or concurrently infected with other pathogens, particularly KoRV. Alternatively, the trypanosome infection may be inducing immunosuppression and therefore potentiating the effects of concomitant diseases (McInnes et al., 2011a).

Mixed infections with up to three *Trypanosoma* spp., including *T. irwini*, *T. gilletti* and *T. copemani* have been reported in koalas (McInnes et al., 2011a; McInnes et al., 2011b). Co-infections with multiple pathogens are common in wildlife and are increasingly recognized as a conservation challenge, as they can result in disease-induced declines and extinctions (de Castro & Bolker, 2005; Joseph et al., 2013). While individual pathogen infections may be tolerated and only present modest clinical signs of disease, interaction effects among multiple pathogens may dramatically alter their pathogenicity. For example, some studies have reported that co-infections with multiple parasite species resulted in more severe clinical disease (Ezeamama et al., 2008).
Further research is required to unravel the biological interactions involved in mixed trypanosome infections and in mixed chlamydial/KoRV and trypanosome infections.

1.3.2.3 *Trypanosoma* infection and the woylie population decline

The woylie is critically endangered and has undergone a rapid population decline of 90% (Thompson et al., 2014b). It has been suggested that trypanosomes may have played a role in this decline. Recent studies provided evidence that *T. copemani* amastigote stages can migrate to and replicate in a range of woylie tissues, including heart, skeletal muscle, oesophagus and tongue, resulting in inflammation and tissue degeneration (Botero et al., 2013; Botero et al., 2016a). These effects may reduce fitness and coordination, making them more susceptible to predation (Botero et al., 2013). This observation has been further supported by a temporal association between *T. copemani* prevalence and declines of the Kingston indigenous woylie population in the Upper Warren region in WA (Thompson et al., 2014b).

A recent study conducted by Hing et al. (2016) revealed a relationship between *T. copemani* infection and the functional efficiency of innate immunity (phagocytosis) in woylies. *T. copemani* infected bettongs had higher faecal cortisol metabolites (FCM) associated with a lower phagocytosis index. However, when the bettongs were trypanosome negative, higher FCM was associated with higher phagocytosis index. This suggests that during periods of *Trypanosoma* parasitaemia, the animals are more vulnerable to the immunosuppressive effects of glucocorticoids. Alternatively, a combination of host stress physiology and infection status may affect the efficiency of leukocyte function (Hing et al., 2016).

Pathological changes in woylies may also occur with mixed infections involving *T. vegrandis* and *T. noyesi*, suggesting a greater immunosuppressive effect or enhanced
pathogenicity with mixed infections (Botero et al., 2013). The finding of a higher prevalence of mixed infections involving *T. copemani*, *T. vegrandis* and *T. noyesi* in a declining woylie population compared to one with stability seems to support this theory (Botero et al., 2013). Conversely, it has been hypothesised that interspecific competition may exist between *T. copemani* and *T. vegrandis*, where an existing *T. vegrandis* infection may moderate the sequential establishment of *T. copemani* (Thompson et al., 2014b). As with koalas, the clinical impact of trypanosome co-infections on woylie health warrants further investigation.

1.3.2.4 **Trypanosomiasis in a little red flying fox**

The first case report of a trypanosome infection associated with clinical disease in bats involved an adult female little red flying fox (*Pteropus scapulatus*), found on the ground in Redcliffe, Qld. The animal presented with icterus and severe anaemia. Necropsy and histological findings were consistent with trypanosome infection of lymphoid tissue and intravascular haemolysis (Mackie et al., 2017). Examination of a blood smear using light microscopy revealed the presence of numerous trypanosomes and molecular analysis revealed the parasite was a potential novel species (Mackie et al., 2017).

1.3.2.5 **Trypanosoma copemani and erythrocyte abnormalities in quokkas**

A recent investigation of the morphological diversity of *T. copemani* in quokkas suggested this parasite species may be associated with erythrocyte abnormalities observed in the blood of infected animals (Austen et al., 2015b). These included the appearance of acanthocytes (star shaped erythrocytes), echinocytes (erythrocytes with many small, evenly spaced thorny projections on the cell membrane), schistocytosis (irregular shaped erythrocytes), dacrocytes (tear drop erythrocytes), microspherocytes (small round dense erythrocytes less than 4 μM with no central pallor) and burst
erythrocytes, which are all cell types typically associated with haemolytic anaemia (Ridak et al., 2012).

1.3.3 Diagnosis

Giemsa or Wright’s stained blood smears are used for detection of trypanosomes by light microscopy (Fig. 1.2 a). Although convenient and readily available, microscopic detection of trypanosomes is relatively insensitive, particularly in chronic stages of infection and in cases of low level parasitaemia (e.g. *T. noyesi*) (Botero et al., 2016b). Various molecular techniques are now available for detection of trypanosomes. For research purposes, electron microscopy is particularly useful for ultrastructural characterisation of the parasites (Fig. 1.2 b).

Polymerase chain reaction (PCR) assays are able to amplify very small amounts of trypanosome DNA in blood. Additionally, PCR combined with Sanger sequencing can be used for species identification. Morphological characterisation is insufficient for species identification, due to overlapping morphometrics between species and polymorphic life cycle stages in vertebrate hosts (Thompson et al., 2013; Austen et al., 2015b). PCR and sequence analysis of partial fragments of the 18S rRNA gene are the most widely used diagnostic method for *Trypanosoma* identification. However, amplification at additional loci, such as the nuclear glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene and the mitochondrial cytochrome B gene, can be adopted for a more robust genetic characterisation (Hamilton et al., 2004; Botero et al., 2016b; Cooper et al., 2017).

Although Sanger sequencing of PCR amplicons, generated by universal primers for the genus *Trypanosoma*, is a fast and sensitive method for species identification, this method can impede the detection of multiple distinct co-amplified genetic variants,
meaning that trypanosome co-infections can remain undetected, particularly when one trypanosome genotype is present at a lower abundance than the other (Carr et al., 2009; Fantin et al., 2013). Thus, studies conducted to date have relied on alternative techniques such as culturing, cloning or species-specific PCR assays to resolve co-infections with trypanosomes (McInnes et al., 2011a; Paparini et al., 2011; Botero et al., 2013). Such methods can, however, be relatively costly and time-consuming when screening a large number of samples and species-specific PCR assays are limited to known species, which greatly limits the possibility of uncovering novel or rare species.

Next-generation sequencing (NGS) allows high-throughput parallelization of sequencing. Although NGS is a well-established method for profiling bacterial communities, with the exception of *Plasmodium* in mosquitoes, relatively few studies have applied this technology in the diagnostics of protozoal infections (Paparini et al., 2015a; Vermeulen et al., 2016; Paparini et al., 2017; Zahedi et al., 2017). There are no previous published studies which have employed an NGS approach to investigate mixed trypanosome infections in wildlife.

### 1.3.4 Treatment and control

There are no specific treatment recommendations for trypanosomiasis in Australian mammals. Supportive care, including blood transfusions, IV fluid therapy, nutritional and thermal support are useful. Additional symptomatic treatments, such as the use of diazepam in koalas exhibiting neurological signs, is recommended (A. Gillett *pers. comm.*).

Prevention and control of trypanosome transmission through limiting exposure to invertebrate vectors is generally not feasible in wildlife. However, monitoring and surveillance to gather baseline data on the prevalence and genetic diversity of
trypanosomes in Australian mammal populations and their vectors is crucial for conservation management, particularly in translocated populations.

1.3.5 Zoonotic potential

Currently, there is no evidence that Australian native trypanosomes are zoonotic. However, a recent study has shown that *T. copemani* is naturally resistant to human serum and therefore may be potentially zoonotic (Austen et al., 2015c).

1.3.6 Biosecurity concerns

The potential negative impact of exotic trypanosomes such as *T. lewisi*, *T. cruzi* and *T. evansi*, if they established and spread within Australian native mammals presents a biosecurity concern (Thompson, 2013; Thompson et al., 2014a). *T. lewisi* is the only exotic species already introduced and found in native mammals (Thompson et al., 2014a).

In 1907, surra-infected camels were imported into Western Australia (WA); fortunately, this infection was diagnosed quickly and *T. evansi* was eradicated prior to establishment (Mackerras 1959). If *T. evansi* had established in Australia, the consequences for Australian native mammals and domestic livestock could have been devastating (Reid et al., 2001; Reid, 2002).

The zoonotic pathogen *T. cruzi* is genetically very similar to the Australian *T. noyesi* (Noyes et al., 1999; Botero et al., 2016b). It has been hypothesised, for example, that the vector of *T. noyesi* could potentially transmit *T. cruzi* from humans (infected immigrants and travellers) to indigenous mammals (Thompson et al., 2014a; Thompson & Thompson, 2015). Experimental *T. cruzi* infections have been demonstrated in the short-beaked echidna (*Tachyglossus aculeatus*) and common brush-tailed possum (Backhouse & Bolliger, 1951), confirming the potential for disease and mortality of
native mammals and the potential for native mammals to act as a reservoir for human infection (Thompson & Thompson, 2015).

Continued surveillance and identification of vectors of Australian trypanosomes are required to ascertain the biosecurity risks of exotic trypanosomes establishing in Australia (Thompson & Thompson, 2015).

1.4 Piroplasms (*Babesia* and *Theileria*)

Piroplasms are tick-borne apicomplexan parasites that infect mammalian and avian erythrocytes. They can cause significant economic losses due to high pathogenicity in domestic production animals and horses worldwide, and severe clinical disease in companion animals (Colwell et al., 2011). Although the clinical significance of piroplasms in Australian native mammals is still relatively unknown, sporadic case reports have suggested that they can be pathogenic (Backhouse & Bolliger, 1957; Barker et al., 1978; Dawood et al., 2013; Kessell et al., 2014; Donahoe et al., 2015).

The Order Piroplasmida includes three genera (*Babesia*, *Theileria* and *Cytauxzoon*) which have been delimited mainly based on parasite life-cycle characteristics. For instance, *Babesia* spp. exhibit transovarial transmission in their tick hosts, in which the parasites migrate to the ovaries and are transmitted vertically from the gravid female to the tick offspring (larvae). In contrast, *Theileria* spp. undergo only transstadial transmission, in which the parasite is passed on from one life stage (‘stadium’) to the next. Furthermore, while *Babesia* spp. only multiply in red blood cells within the vertebrate hosts, *Theileria* spp. undergo extra-erythrocytic schizogony in lymphocytes or macrophages, prior to invading erythrocytes (Uilenberg, 2006; Sivakumar et al., 2014).
However, a recent phylogenetic study of concatenated mitochondrial and 18S rDNA sequences as well as cytochrome c oxidase subunit I (cox1) amino acid sequence identified five distinct *Piroplasmida* groups, including four previously identified clades (*B. microti* group, *Babesia* sensu stricto, *Theileria equi* and *Babesia* sensu latu group), while supporting the integration of *Theileria* and *Cytauxzoon* species into a single fifth taxon (Schreeg et al., 2016).

Since previous research has concentrated mainly on parasites of recognised clinical importance, phylogenetic analysis of piroplasms remains relatively incomplete for wildlife species globally. In Australia, there are 10 formally named species of piroplasms (*Babesia* and *Theileria*) recorded in native mammals, and genetic data from four un-named marsupial-derived piroplasms. These species have been classified based on morphological analysis and/or molecular analysis. In these studies, relatively scarce genetic information available for Australian piroplasms, along with significant limitations associated with morphology-based identifications, have contributed to a confused taxonomic systematics of the Piroplasmida, characterized by soft polytomies (unresolved relationships), paraphyly/polyphyly and contrasting tree topologies (Paparini et al., 2012b; Paparini et al., 2015b). More investigation into genetic characterisation and life-cycles is warranted to further understand the evolution of the Piroplasmida, particularly of Australian piroplams.

The species’ names of piroplasms documented in Australian mammals to date, as well as their known vertebrate host range, geographic distribution and overall clinical significance are presented in Table 1.2.
Table 1.2. Piroplasms (*Babesia* and *Theileria*) of Australian native mammals

<table>
<thead>
<tr>
<th>Piroplasm species</th>
<th>Host (s)</th>
<th>Origin(s)/Distribution</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Babesia tachyglossi</em> / <em>Theileria tachyglossi</em> 1,2,3,21</td>
<td>Short-beaked echidna (&lt;i&gt;Tachyglossus aculeatus&lt;/i&gt;)</td>
<td>NSW, Qld, WA</td>
<td>Associated with a mortality event involving 12 captive individuals in NSW</td>
</tr>
<tr>
<td><em>T. ornithorhynchi</em> 3,5,6,8,18,20,21</td>
<td>Platypus (&lt;i&gt;Ornithorhynchus anatinus&lt;/i&gt;)</td>
<td>NSW, Qld, Tas.</td>
<td>High parasitaemia associated with dehydration, anorexia and fatal haemolytic anaemia</td>
</tr>
<tr>
<td><em>B. macropus</em> 12,17,19</td>
<td>- Agile wallaby (&lt;i&gt;Macropus agilis&lt;/i&gt;)&lt;br&gt;- Eastern grey kangaroo (&lt;i&gt;M. giganteus&lt;/i&gt;)</td>
<td>NSW, Qld</td>
<td>Severe clinical disease</td>
</tr>
<tr>
<td><em>B. thylacis</em> * 3,7</td>
<td>- Northern brown bandicoot (&lt;i&gt;Isodon macrourus&lt;/i&gt;)&lt;br&gt;- Northern quoll (&lt;i&gt;Dasyurus hallucatus&lt;/i&gt;)&lt;br&gt;- Southern brown bandicoot (&lt;i&gt;I. obesulus&lt;/i&gt;)</td>
<td>Qld, WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>B. vogeli</em> 10,14</td>
<td>- Dingo (&lt;i&gt;Canis lupus dingo&lt;/i&gt;)&lt;br&gt;- Dingo/ domestic dog (&lt;i&gt;C. lupus familiaris&lt;/i&gt;) hybrids</td>
<td>NT, WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Babesia sp.</em> 4,9,10</td>
<td>- Agile antechinus (&lt;i&gt;Antechinus agilis&lt;/i&gt;)&lt;br&gt;- Brown antechinus (&lt;i&gt;A. stuartii&lt;/i&gt;)&lt;br&gt;- Proserpine rock-wallaby (&lt;i&gt;Petrogale persephone&lt;/i&gt;)</td>
<td>Vic</td>
<td>Severe clinical disease</td>
</tr>
<tr>
<td>Piroplasm species</td>
<td>Host (s)</td>
<td>Origin(s)/ Distribution</td>
<td>Clinical significance</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------------------------------------</td>
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<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Babesia sp. 15</td>
<td>Woylie (or brush-tailed bettong) <em>B. penicillata</em></td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>T. brachyuri 11</td>
<td>Quokka <em>Setonix brachyurus</em></td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>Theileria sp. isolate K1</td>
<td>Burrowing bettong <em>B. lesueur</em></td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>T. fuliginosa 11</td>
<td>Western grey kangaroo <em>M. fuliginosus</em></td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>T. gilberti 13</td>
<td>Gilbert’s potoroo <em>Potorous gilbertii</em></td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>T. penicillata 11,16</td>
<td>Woylie or brush-tailed bettong</td>
<td>WA</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

- Long-nosed bandicoot *(Perameles nasuta)*
- Long-nosed potoroo *(P. tridactylus)*
- Northern brown bandicoot

T. peramelis *3*  
Qld  
Anaemia without apparent clinical illness

Theileria sp. 13  
Long-nosed potoroo  
WA  
Unknown


*Species classification based on morphological characterisation only.
1.4.1 Epidemiology

The true prevalence of piroplasms in Australian native mammals is unknown although there have been recent studies of limited populations in geographically disparate regions. A high prevalence of *T. gilberti* in free-ranging populations of the Gilbert’s potoroo (*Potorous gilbertii*) (100%, 16/16) and *T. penicillata* in woylies (80.4%, 123/153) from WA have been reported (Lee et al., 2009; Rong et al., 2012). In another study conducted on captive and free-ranging marsupial populations in WA (Paparini et al., 2012b), the overall detection rate of piroplasm-infected animals was 7.1% (8/113). Of these, a novel *Babesia* was identified in seven free-ranging woylies and a single *Theileria* positive sample was identified from one of three captive burrowing bettongs (*B. lesueur*) (Paparini et al., 2012b). Another study focusing on the health of free-ranging eastern bettongs (*B. gaimardi*) captured in Tasmania (Tas) and translocated to the Australian Capital Territory (ACT), examined blood films by microscopy and intraerythrocytic piroplasms were observed in 6.7% (4/60) of the animals (Portas et al., 2014).

Piroplasms in native mammals have been recorded in most states and territories of Australia (Table 1.2). Ticks are believed to be the main vectors, as they are ubiquitous and parasitize all vertebrate hosts of *Babesia* and *Theileria* species recorded to date. Ticks collected from infected animals have also been positive for piroplasms using molecular methods. In this context, native *Haemaphysalis* spp. and *Ixodes* spp. seem likely vector candidates (Dawood et al., 2013).

1.4.2 Pathogenesis and clinical significance of piroplasmosis in Australian native mammals

Although most piroplasms reported in Australian mammals appear non-pathogenic, there are some examples of morbidity and mortality associated with infection.
Haemolytic anaemia and thrombocytopenia are recognised as the most typical pathology of piroplasmosis. These may be the result of the physical effects of parasites on cells or the activation of an immune response which leads to cell destruction by macrophages (Clark et al., 2004). Clinical signs of piroplasmosis may include lethargy, reduced mentation, diarrhoea and neurological signs and these signs may vary with species of parasite and host.

Interestingly, the pathogenesis of *B. macropus* in macropods appears to have similarities with that of *B. bovis* in cattle, characterised by sequestering of infected erythrocytes in the capillaries of organs, leading to low peripheral parasitaemia, disseminated intravascular coagulation and severe cerebral babesiosis (Dawood et al., 2013).

The following information relates to documented cases of clinical signs potentially associated with piroplasm infections.

### 1.4.2.1 *Babesia/Theileria tachyglossi* and short-beaked echidna mortality

Piroplasms (*B. tachyglossi* and *T. tachyglossi*) found in short-beaked echidnas were originally described based solely on microscopy (Priestley, 1915; Backhouse & Bolliger, 1957, 1959; Mackerras, 1959). At the time of the original reports, the genera *Babesia* and *Theileria* had not yet been defined, in regard to the existence of schizonts and/or transstadial vs. transovarial transmission. Molecular data suggests that only a single piroplasm species, believed to be *T. tachyglossi*, infects echidnas (Šlapeta et al., 2017).

Infection with piroplasms was implicated in the deaths of 12 captive short-beaked echidnas (Backhouse & Bolliger, 1957). Subsequently, there have been no further reports of pathological effects of piroplasms in echidnas despite these parasites being frequently observed in blood films (L. Vogelnest *pers. comm.*). Monotreme populations
in Australia remain relatively understudied with respect to the prevalence and clinical impact of piroplasm infection.

1.4.2.2 **Anaemia in bandicoots associated with Theileria peramelis**

Although natural infections with *T. peramelis* have been recorded in southern brown (*Isoodon obesulus*) and long-nosed bandicoots (*Perameles nasuta*), there is only one report associating experimental infection with *T. peramelis* and anaemia in these species. The anaemia observed was strongly regenerative, characterised by increased anisocytosis and immature erythrocytes (Mackerras, 1959).

1.4.2.3 **Babesia infection and fatal anaemia in antechinuses**

Infection with a *Babesia* sp. has been associated with clinical piroplasmosis in the brown antechinus (*Antechinus stuartii*) and male agile antechinuses (*A. agilis*) undergoing physiological stress in the post-mating period. These animals presented with decreased PCV, haemoglobinuria and haemosiderosis of the lung and spleen (Cheal et al., 1976; Barker et al., 1978).

1.4.2.4 **Theileria ornithorhynchi and fatal anaemia in platypuses**

Although *T. ornithorhynchi* is generally considered non-pathogenic, this parasite has been associated with fatal haemolytic anaemia in an orphaned juvenile platypus. The animal had concurrent fungal dermatitis and a heavy tick burden (Kessell et al., 2014). Association of *T. ornithorhynchi* with disease is further supported by a recent report of high (10–15%) parasitaemia in three diseased male wild platypuses (two juvenile and one sub-adult) (Šlapeta et al., 2017). One of them was also infected with *Eimeria* sp. The animals were dehydrated and anorexic. Histopathology revealed severe suppurative enteritis and active spleen with increased erythrophagocytosis (Šlapeta et al., 2017).
1.4.2.5  *Babesia macropus* and severe babesiosis in macropods

Although subclinical infection with *Babesia* spp. appears to be relatively common in macropods, *B. macropus* infection has been associated with a syndrome of haemolytic anaemia and debility in hand-reared and free-ranging juvenile eastern grey kangaroos, agile wallabies (*M. agilis*), red-necked wallabies (*M. rufogriseus*) and swamp wallabies (*Wallabia bicolour*). Clinical signs included severe pallor, lethargy, polydipsia, polyuria, neurological signs and death (Vogelnest & Portas, 2008; Dawood et al., 2013; Donahoe et al., 2015). PCV of less than 10% are commonly observed in affected animals, which may also be hypoproteinaemic with total protein levels as low as 30 g/L (Vogelnest & Portas, 2008).

Other variable, clinical pathological findings associated with this disease included thrombocytopenia, neutropenia, hyperamylasaemia, azotaemia and bilirubinaemia. The neurological signs observed in some kangaroos may be related to intravascular sequestration of parasitized erythrocytes within the central nervous system. Potential pathogenic mechanisms include hypoxia, inflammatory cytokine release following obstructive sequestration of erythrocytes and endothelial damage (Donahoe et al., 2015). Necropsy findings included diffuse pallor of the carcass and visceral organs, thin watery blood, widespread petechiae, ecchymosis, tissue oedema, splenomegaly and generalised lymphadenomegaly (Donahoe et al., 2015).

As most of the infected kangaroos were captive/hand-reared, it is likely that the stress of handling, transportation and captivity contributed to the development of disease. Furthermore, kangaroos hand-reared by carers lack the relevant maternal antibodies and also have less exposure to naturally occurring parasites compared to free-ranging kangaroos, therefore rendering them more susceptible to infection (Donahoe et al.,
Alternatively, it is possible that the disease was more likely to be detected in captive animals than from free-ranging animals.

1.4.3 Diagnosis

Although microscopy has frequently been used as the primary method for investigation of piroplasms, this method alone lacks sensitivity, particularly in chronic or subclinical cases (Clark et al., 2004). Moreover, morphological features within intra-erythrocytic piroplasms are undistinguishable among different genus and species (Homer et al., 2000). Schizonts of *Theileria* spp., present in leukocytes, may also be very difficult to find.

Additionally, piroplasm morphology in Australian mammals is highly pleomorphic, with intra-erythrocytic organisms characterised as ring-shaped trophozoites (Fig. 1.4a) and/or pairs or tetrads of pyriform shaped merozoites (Fig. 1.4b). Even though the infection typically affects less than 1% of erythrocytes (Clark et al., 2004), piroplasms may exhibit high parasitaemia, as observed for *T. gilberti* in the Gilbert’s potoroo (Lee et al., 2009) (Fig. 1.4a).
Figure 1.4. Piroplasms in Australian mammal species a) Ring-shaped trophozoites in the blood of a Gilbert’s potoroo exhibiting a high *T. gilberti* parasitaemia. b) Photomicrograph of a tetrad of pyriform-shaped piroplasms isolated from the blood of a platypus and identified by PCR as *T. ornithorhynchi*. Scale bars = 10 µm. Images courtesy of Prof. Peter Irwin.

Molecular methods are not only significantly more sensitive than microscopy, but also required for confirmation of genus and species (Schnittger et al., 2003; Clark et al., 2004). PCR assays targeting the 18S rRNA gene are the most widely used molecular method for the detection and characterisation of piroplasms in Australian mammals (Paparini et al., 2012b; Dawood et al., 2013). In addition, a NGS-based assay was recently developed to study the diversity of apicomplexan parasites (including piroplasms) in platypuses and echidnas (Šlapeta et al., 2017).

1.4.4 Treatment and control

While a wide range of antiprotozoal drugs has been used for the treatment of piroplasmosis in domestic animals, there is currently no robust scientific evidence of effectiveness of these drugs in Australian mammals, or awareness of potential toxic effects. There are occasional reports of parenteral treatment of infected native species
with imidocarb dipropionate; however, response to this treatment remains anecdotal and unevaluated (Vogelnest & Portas, 2008; Dawood et al., 2013; Kessell et al., 2014; Donahoe et al., 2015).

Suggested treatment regimens in native species include provision of supportive care, including blood transfusions, intravenous fluids, improved nutrition and warmth if the animal is hypothermic (Vogelnest & Portas, 2008).

As with trypanosomes, prevention of piroplasm transmission by vector control is not generally feasible in wildlife. Administration of prophylactic doses of anti/protozoal medication has been suggested for macropods before and after translocation to prevent development of clinical babesiosis (Donahoe et al., 2015). Awareness of the prevalence and genetic diversity of piroplasms is crucial for appropriate conservation management and translocation strategies.

1.4.5 Zoonotic potential

The first autochthonous case of human babesiosis reported in Australia was diagnosed in 2010, and the aetiological agent was identified as B. microti (Senanayake et al., 2012). Even though several Babesia spp. have been isolated from Australian mammals, there is currently no reservoir identified for this zoonotic Babesia in Australia. There is no scientific evidence that Theileria spp. infecting Australian mammals are zoonotic.

1.5 Hepatozoon spp.

The apicomplexan genus Hepatozoon includes more than 300 species which infect all classes of vertebrates (Smith, 1996; Baneth, 2011). While infections in most mammalian hosts are typically subclinical, species that infect canid and felid carnivores (H. americanus, H. canis and H. felis), known to occur in Africa, Asia, Europe, South
America, and the USA (Baneth, 2011), are of veterinary importance (Modry et al., 2017).

Infection of the vertebrate intermediate host usually occurs by ingestion of an infected tick (definitive host), although it can also occur by vertical transmission or by predation. In cases of infection via ingesting an infected invertebrate host, the sporozoites released from sporocysts within oocysts, enter the circulation and migrate to various organs where they undergo merogony. When merozoites are released from mature meronts into the bloodstream, they enter the blood cells and transform into gamonts, which are later ingested with the blood meal by a competent vector (Modry et al., 2017; O'Donoghue, 2017).

The genus *Hepatozoon* has been described as paraphyletic (Barta et al., 2012; Karadjian et al., 2015). Recent studies suggest the taxonomy of *Hepatozoon* is unsettled and different phylogenetic clades probably separate genera (Modry et al., 2017).

Several species of *Hepatozoon* have been identified from Australian marsupials to date (Table 1.3), most of which are based on morphometric descriptions and host species only. More recently, a study reported for the first time, several *Hepatozoon* genotypes from southern brown bandicoots (Wicks et al., 2006). The organisms were also morphologically characterised; however, due their morphometric similarities to *H. peramelis* (Welsh & Dalyell, 1909; Mackerras, 1959), for which no genetic data was available, the species identity could not be confirmed (Wicks et al., 2006).
### Table 1.3. *Hepatozoon* spp. of Australian native mammals

<table>
<thead>
<tr>
<th>Hepatozoon sp.</th>
<th>Host (s)</th>
<th>Origin/Distribution</th>
<th>Clinical significance</th>
</tr>
</thead>
</table>
| *Hepatozoon dasyuri*<sup>5</sup> | -Native cat (*Dasyurus quoll*)  
- Eastern quoll (*D. viverrinus*) | Qld | Unknown |
| *H. dasyuroides*<sup>6</sup> | Crested-tailed marsupial rat (*D. byrnei*) | Qld | Unknown |
| *H. muris*<sup>1,2</sup> | - Allied rat (*Rattus assimilis*)  
- Dusky Field rat (*R. sordidus*)  
- Bush rat (*R. fuscipes*) | Qld, NSW, WA | Unknown |
| *H. peramelis*<sup>4,6</sup> | - Southern-brown bandicoot (*Isoodon obesulus*)  
- Long-nosed bandicoot (*Perameles nasuta*) | NSW, Qld | Unknown |
| *H. petauri*<sup>3</sup> | - Sugar-glider (*Petaurus breviceps*)  
- Squirrel-glider (*P. norfolcensis*) | NSW | Unknown |
| *H. pseudocheiri*<sup>6</sup> | Ring-tail possum (*Pseudocheirus laniginosus*) | Data not available | Unknown |
| *H. tachyglossi*<sup>9,11</sup> | Short-beaked echidna (*Tachyglossus aculeatus*) | Vic | Unknown |
| *Hepatozoon sp.*<sup>7</sup> | Eastern barred bandicoots (*P. gunnii*) | Tas. | Unknown |
| *Hepatozoon sp.*<sup>10</sup> | Southern brown bandicoot | WA | Unknown |
| *Hepatozoon sp.*<sup>8</sup> | Herbert River ring-tail possum (*Pseudochirulus herbertensis*) | Qld | Unknown |
| *Hepatozoon sp.*<sup>8</sup> | Bush rat | Data not available | Unknown |

<sup>1</sup>Balfour 1906; <sup>2</sup>Cleland 1906; <sup>3</sup>Welsh & Barling 1909; <sup>4</sup>Welsh & Dalyell 1909; <sup>5</sup>Welsh et al. 1910; <sup>6</sup>Mackerras 1959; <sup>7</sup>Bettiol et al. 1996; <sup>8</sup>O’Donogue & Adlard 2000; <sup>9</sup>Clark et al. 2005; <sup>10</sup>Wicks et al. 2006; <sup>II</sup>Ploeg et al. 2008 /  
*Species classification based on morphological characterisation only.*
1.5.1 Epidemiology

Little is known about the epidemiology of *Hepatozoon* spp. and their vectors in Australian mammals. However, this parasite seems to have a widespread distribution in the Australian continent (Welsh & Dalyell, 1909; Mackerras, 1959; Bettiol et al., 1996; O'Donoghue & Adlard, 2000; Wicks et al., 2006).

To date, only two studies have reported the prevalence of *Hepatozoon* in Australian mammals to date, which ranged from 25% (55/220) in bandicoots from Tasmania (Bettiol et al., 1996) and 58.1% (18/31) in bandicoots from WA (Wicks et al., 2006).

1.5.2 Pathogenesis and clinical significance of hepatozoonosis

Some *Hepatozoon* spp. that infect canid and felid carnivores may cause a range of clinical signs from anaemia, lethargy, and cachexia to severe cardiac and skeletal muscle infection (myositis). *Hepatozoon* infection may also compromise the host immune status, thus predisposing the host to infection with other pathogens. Similarly, immunocompromised individuals, such as those with concurrent immunosuppressive viral infections, may be more susceptible to severe *Hepatozoon* infection (Cunningham & Yabsley, 2012).

There is no evidence of clinical disease associated with *Hepatozoon* infection in Australian mammals.

1.5.3 Diagnosis

Most studies to date have been confined to microscopic detection of *Hepatozoon* gamonts within red blood cells or other peripheral blood cells of vertebrate hosts. In mammalian hosts, the gamonts are mostly present in white blood cells (Modry et al., 2017). The traditional direct diagnostic methods for rapid detection of *Hepatozoon* include thick and thin blood smears and buffy coat smears (Modry et al., 2017).
Gamonts are somewhat oval to elongate, with a pale to basophilic cytoplasm and may contain sparse fine basophilic granules (Fig 1.5). Similar to trypanosomes and piroplasms, microscopic methods are not sensitive for detecting *Hepatozoon* in animals with low parasitaemia (Cunningham & Yabsley, 2012).

![Figure 1.5](image1.png)

**Figure 1.5.** *Hepatozoon* sp. in an erythrocyte from the blood of a southern brown bandicoot *Isoodon obesulus*. Scale bar = 10 µm. (Bettiol et al., 1996).

To date, similar to other blood-borne protists, molecular diagnostic methods developed for *Hepatozoon* have relied mainly on the amplification of 18S rRNA gene fragments. However, studies on reptilian *Hepatozoon* have used ITS-1 as an additional or alternative target for PCR assays and phylogenetic analyses (Boulianne et al., 2007). Two quantitative PCRs (qPCRs) assays targeting the conserved 18S rRNA gene have been developed to estimate the prevalence and intensity of *Hepatozoon* infection in carnivore species (Criado-Fornelio et al., 2007; Li et al., 2008).
1.5.4 Treatment and control

Antiprotozoal drugs, particularly imidocarb dipropionate, are used to treat hepatozoonosis in dogs. In Australia, given there is no evidence of clinical disease associated with *Hepatozoon* infection in native mammals, no protocols for treatment and control have been established.

1.5.5 Zoonotic potential

*Hepatozoon* spp. are not known to be zoonotic.

1.6 *Cryptosporidium* spp.

*Cryptosporidium* species are ubiquitous protozoan parasites that infect a broad range of hosts including humans, domestic and wild animals worldwide, causing asymptomatic or mild to severe gastrointestinal disease in their host species (Monis & Thompson, 2003; Xiao, 2010; Ryan et al., 2014). Infection occurs via the faecal/oral route, either by direct ingestion of infective forms or by ingestion of contaminated food or water. Due to the wide host range and environmental persistence of this parasite, cryptosporidiosis can be zoonotic and associated with foodborne and waterborne outbreaks. Currently, 34 species are regarded as valid and of these, *C. hominis* and *C. parvum* are responsible for the majority of infections in humans (Ryan et al., 2016).

The infectious form of *Cryptosporidium* is the oocyst. Overall, following ingestion by the host, sporozoites are released from oocysts and infect host gut epithelial cells. The sporozoites then differentiate into trophozoites, which differentiate into type I meronts. Merozoites produced by type I meronts are released from the host cell and can infect other host cells. These merozoites can then either undergo asexual reproduction by differentiating into trophozoites, or initiate the sexual cycle by differentiating into type II meronts which give rise to gamonts (macro and micro). Oocysts are produced
following sexual reproduction. Two types of oocysts are produced; thick-walled oocysts, which are the infectious form found in the environment, and thin-walled oocysts, which cause auto-infection (Monis & Thompson, 2003).

Until recently, the taxonomy of Cryptosporidium was confused as, initially, the parasite was classified with the coccidians and individual species were recognized based on oocyst morphology, presumed host specificity and cross-transmission studies only (O'Donoghue, 1995). An increasing number of studies have, however, examined the prevalence of Cryptosporidium using genotyping techniques and its relationship to other apicomplexan parasites (Sulaiman et al., 2000; Kvac et al., 2014; Ryan et al., 2016), therefore improving the current understanding of the genetic diversity and phylogenetic relationships with other apicomplexans and among Cryptosporidium spp. As a result, Cryptosporidium has been formally transferred from the Coccidia, to the gregarines (a primitive apicomplexan group) and into a new subclass, Cryptogregaria (Cavalier-Smith, 2014).

In Australia, several species and novel genotypes of Cryptosporidium have been recorded in native mammals (Table 1.4)
<table>
<thead>
<tr>
<th>Cryptosporidium sp.</th>
<th>Host(s)</th>
<th>Origin/Distribution</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryptosporidium fayeri&lt;sup&gt;1,2,6,8,12,13,14,15,16,17,19,23,25&lt;/sup&gt;</td>
<td>-Southern brown bandicoot (<em>Isoodon obesulus</em>)&lt;br&gt;-Western-barred bandicoot (<em>Perameles bougainville</em>)&lt;br&gt;-Koala (<em>Phascolarctos cincerus</em>)&lt;br&gt;-Red kangaroo (<em>Macropus rufus</em>)&lt;br&gt;-Eastern grey kangaroo (<em>M. giganteus</em>)&lt;br&gt;-Western grey kangaroo (<em>M. fuliginosus</em>)&lt;br&gt;-Yellow-footed rock-wallaby (<em>Petrogale xanthopus</em>)&lt;br&gt;-Brush-tailed rock-wallaby (<em>P. penicillata</em>)</td>
<td>NSW, WA, Vic</td>
<td>Unknown</td>
</tr>
<tr>
<td>C. macropodum&lt;sup&gt;5,6,9,10,11,18,19,21,23&lt;/sup&gt;</td>
<td>-Red kangaroo&lt;br&gt;-Eastern grey kangaroo&lt;br&gt;-Western grey kangaroo&lt;br&gt;-Swamp wallaby (<em>Wallabia bicolor</em>)</td>
<td>NSW, WA, Vic</td>
<td>Unknown</td>
</tr>
<tr>
<td>C. cuniculus&lt;sup&gt;24&lt;/sup&gt;</td>
<td>Eastern grey kangaroo</td>
<td>Vic</td>
<td>Unknown</td>
</tr>
<tr>
<td>C. meleagris&lt;sup&gt;25&lt;/sup&gt;</td>
<td>Brush-tailed rock-wallaby</td>
<td>NSW</td>
<td>Unknown</td>
</tr>
<tr>
<td>C. muris&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Bilby (<em>Macrotis lagotis</em>)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>C. hominis&lt;sup&gt;18,26,27,28&lt;/sup&gt;</td>
<td>-Southern brown bandicoot&lt;br&gt;-Common brush-tailed possum (<em>Trichosurus vulpecula</em>)&lt;br&gt;-Eastern grey kangaroo&lt;br&gt;-Brush-tailed rock-wallaby&lt;br&gt;-Wild dingo (<em>Canis lupus dingo</em>)&lt;br&gt;-Little-red flying fox (<em>Pteropus conspicillatus</em>)</td>
<td>NSW, Qld</td>
<td>Unknown</td>
</tr>
<tr>
<td>Cryptosporidium sp.</td>
<td>Host (s)</td>
<td>Origin/Distribution</td>
<td>Clinical significance</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------</td>
<td>---------------------</td>
<td>-----------------------</td>
</tr>
</tbody>
</table>
| *C. parvum*³,⁸,¹⁸,²²,²⁷ | -Eastern grey kangaroo  
-Long-nosed bandicoot (*P. nasuta*)  
-Southern brown bandicoot  
-Common brush-tailed possum  
-Wallaby (no species identification) | NSW | Unknown |
| *C. tyzzeri*³ | -Wild house mouse (*Mus domesticus*)  
-Large-footed mouse-eared bat (*Myotus adversus*) | NSW, Vic | Unknown |
| Cryptosporidium sp. kangaroo genotype 1⁹ | Western grey kangaroo | WA | Unknown |
| Cryptosporidium sp. brushtail possum genotype 1⁸ | Common brush-tailed possum | NSW | Unknown |
| Cryptosporidium sp. mouse genotype II⁷ | Wild house mouse | Qld | Unknown |
| Cryptosporidium sp. bat genotypes VIII-XI ²⁶ | Grey-headed flying fox (*P. poliocephalus*) | NSW, Qld | Unknown |
| Cryptosporidium sp. ²⁰ | Black rat (*Rattus rattus*) | NT | Unknown |

¹O’Donoghue, 1995; ²Morgan et al. 1997; ³Morgan et al., 1999; ⁴Warren et al., 2003; ⁵Power et al., 2004; ⁶Power et al., 2005; ⁷Foo et al., 2007; ⁸Hill et al., 2008; ⁹McCarthy et al. 2008; ¹⁰Power, 2008; ¹¹Power & Ryan, 2008; ¹²Ryan et al., 2008; ¹³Yang et al., 2008; ¹⁴Power et al., 2009; ¹⁵Power, 2010; ¹⁶Waldron et al., 2010; ¹⁷Feng et al., 2011; ¹⁸Ng et al., 2011; ¹⁹Yang et al., 2011; ²⁰Paparini et al., 2012; ²¹Ryan & Power, 2012; ²²Dowle et al., 2013; ²³Nolan et al., 2013; ²⁴Koehler et al., 2014; ²⁵Vermeulen et al., 2015; ²⁶Schiller et al., 2016; ²⁷Zahedi et al., 2016a; ²⁸Zahedi et al., 2016b.

1.6.1 Epidemiology

*Cryptosporidium* spp. and genotypes recorded in native marsupials to date have a widespread distribution in Australia (Table 1.4). Reported prevalences in these animals ranged from 1.9%–19.3% (Power et al., 2003; Power et al., 2004; Cox et al., 2005; Power et al., 2005; McCarthy et al., 2008; Ng et al., 2011; Yang et al., 2011; Ryan &
Infections with *Cryptosporidium* spp. in native and exotic rodents have been reported in Qld, Vic and NT at prevalences ranging from 7.6% to 8.2% (Morgan et al., 1999; Foo et al., 2007; Paparini et al., 2012a). A *Cryptosporidium* prevalence of 3.2% in faecal samples from flying foxes, originated from urban and rural locations in NSW and Qld was reported using PCR screening (Schiller et al., 2016).

1.6.2 Pathogenesis and clinical significance of cryptosporidiosis in Australian native mammals

The mechanisms that contribute to disease caused by *Cryptosporidium* are not totally understood, but are clearly multifactorial and involve both parasite and host immune factors as well as the gut environment (Bouzid et al., 2013; Ryan et al., 2016; Certad et al., 2017). Currently > 25 putative virulence factors have been identified which are proposed to be involved in aspects of host-pathogen interactions from adhesion and locomotion to invasion and proliferation (Bouzid et al., 2013). For example, *Cryptosporidium* inhibits cell apoptosis at the trophozoite stage, but promotes this process at the sporozoite and merozoite stages and several molecules, such as phospholipases, proteases, and haemolysins, may cause cellular damage, enhancing fluid secretion from the crypts and supporting diarrhoea due to active secretion and malabsorption (Certad et al., 2017).
Little is known about the pathogenicity of *Cryptosporidium* in wildlife. There are numerous *Cryptosporidium* species and genotypes that appear to be adapted to particular species of wildlife with little evidence of any clinical impact, which suggest well-balanced host-parasite relationships, however much more research is required in this area. Although zoonotic variants of *Cryptosporidium* have also been identified in faecal samples of marsupials, no evidence of associated clinical disease has been reported (Ryan & Power, 2012; Thompson & Ash, 2016).

1.6.3 Diagnosis

There are many challenges associated with *Cryptosporidium* detection and diagnosis. The use of different diagnostic methods and the inconsistent application of typing techniques can make direct comparisons difficult or even impossible between clinical, veterinary and environmental testing or between different regions or countries (Ryan et al., 2016).

Microscopic detection is usually conducted using stains and/or fluorescent antibodies (IFA). As stated previously in this chapter, this method can be time-consuming and lacks sensitivity. Further, most species of *Cryptosporidium* are morphologically indistinguishable and can only be identified using molecular tools (Ryan & Power, 2012).

Molecular techniques have allowed for specific and sensitive detection and differentiation of *Cryptosporidium* spp. for clinical diagnosis and environmental surveillance. The most widely used molecular markers for identification and typing of *Cryptosporidium* species are the 18S rRNA locus and the 60-kDa glycoprotein (gp60) gene, respectively (Xiao, 2010; Zahedi et al., 2016b).
Real-time or quantitative PCR (qPCR) assays, have also been used to quantitate the numbers of *Cryptosporidium* oocysts present in human and animal faecal and water samples exhibiting high sensitivity and specificity (Hadfield et al., 2011; Yang et al., 2013; Yang et al., 2014). Multiplex qPCR methodologies have also been developed to detect mixed infections with *Cryptosporidium* and other enteric pathogens such as *Giardia* and *Entamoeba* (Nurminen et al., 2015). More recently, NGS technology was employed in the investigation of within-host diversity of *Cryptosporidium* spp. (Grinberg et al., 2013; Zahedi et al., 2017).

1.6.4 Treatment and control

Currently, nitazoxanide (NTZ) is approved for treatment of cryptosporidiosis in children and immunocompetent adults in the U.S.A., however NTZ is not effective without an appropriate immune response and is therefore ineffective for the treatment of immunocompromised individuals (Ryan et al., 2016). There are no specific treatment recommendations for cryptosporidiosis in Australian mammals and successful prevention and management can only be achieved through a deep understanding of the routes of transmission, sources of contamination (human and animal) and prevalence of infections (Ryan et al., 2016).

1.6.5 Zoonotic potential and One Health implications

Humans, wildlife and domestic livestock all potentially contribute *Cryptosporidium* to surface waters. Human encroachment into natural ecosystems has led to an increase in interactions between humans, domestic animals and wildlife populations. An increasing number of zoonotic diseases and spill over/back of zoonotic pathogens are a consequence of these interactions. In this context, from a One Health perspective, the epidemiology of zoonotic species of *Cryptosporidium* in wildlife species, particularly
Australian native mammals, constitutes an important research requirement (Zahedi et al., 2016a; Zahedi et al., 2016b).

1.7 *Giardia* spp.

Species in the genus *Giardia* are intestinal protozoan parasites which can cause diarrhoeal illness in animals and humans worldwide. Its simple life-cycle involving an environmentally resistant cyst, provides ample opportunities for the parasite to be transmitted directly from one infected individual to another, or indirectly through contamination of the environment or food (Thompson, 2000). Giardiasis, the disease caused by *Giardia*, is the most frequently diagnosed waterborne disease and along with *Cryptosporidium*, is one the major public health concerns of water utilities in both developed and developing nations (Thompson, 2004).

The morphological features of *Giardia* are characteristic and distinctive. The non-encysted, motile trophozoite is bilaterally symmetrical, piriform to ellipsoidal in shape with a convex dorsal surface and large unique adhesive or ‘sucking disc’ on the ventral surface. It is binucleate, with four pairs of flagella and a pair of distinctive median bodies (Thompson, 2004) (Fig 1.6)
Figure 1.6. Giemsa-stained trophozoite of *Giardia duodenalis* showing multiple flagella, nuclei and median bodies (Thompson, 2004).

The taxonomy of *Giardia* has been a matter of controversy for a number of years, particularly due to the morphological similarity of the described species and doubts over the validity of host specificity as a criterion for taxonomic recognition. There are currently seven recognised species of *Giardia* (Thompson, 2004; Hillman et al., 2016). Of these, *G. duodenalis* (syn. *G. lamblia* and *G. intestinalis*) is known to infect multiple host species, including most mammals and humans (Caccio & Ryan, 2008).

Molecular studies have demonstrated that *G. duodenalis* is a species complex comprising at least eight major genetically and phenotypically distinct assemblages with assemblages A and B in humans and other mammals; C and D in dogs and other canids; E mainly in ungulates, F mainly in cats; G in rats and mice; and H in marine mammals (Thompson and Ash, 2016). Assemblages A and B have also been recorded in a range of domestic and wild animals worldwide, including Australian marsupials (Monis & Thompson, 2003; Thompson et al., 2008; Thompson et al., 2010; Thompson & Ash, 2016). Wildlife also harbour their own apparently host-adapted species of *Giardia,*
including *G. peramelis*, in the quenda, southwest WA subspecies of southern brown bandicoot (*Isoodon obesulus fusciventer*) (Adams et al., 2004; McCarthy et al., 2008; Hillman et al., 2016). Species and assemblages of *Giardia* recorded in Australian mammals to date are presented in table 1.5.

### Table 1.5. *Giardia* spp. identified in Australian native mammals

<table>
<thead>
<tr>
<th><em>Giardia</em> spp.</th>
<th>Host(s)</th>
<th>Origin/Distribution</th>
<th>Clinical significance</th>
</tr>
</thead>
</table>
| *Giardia duodenalis* (Assemblage A)<sup>1,6,7,8</sup> | - Southern brown bandicoot (*Isoodon obesulus*)  
- Common planigale (*Planigale maculata*)  
- Kangaroo (no species identification)  
- Red kangaroo (*Macropus rufus*)  
- Western grey kangaroo (*M. fuliginosus*)  
- Parma wallaby (*M. parma*)  
- Tammar wallaby (*M. eugenii*)  
- Quokka (*Setonix brachyurus*)  
- Yellow-footed rock-wallaby (*Petrogale xanthopus*)  
- Swamp wallaby (*Wallabia bicolor*)  
- Brush-tailed rock-wallaby (*P. penicillata*)  
- Koala (*Phascolarctos cinereus*)  
- Rufous bettong (*Aepyprymnus rufescens*)  
- Long-nosed potoroo (*Potorous tridactylus*)  
- Southern hairy-nosed wombat (*Lasiorhinus latifrons*)  
- Common brush-tailed possum (*Trichosurus vulpecula*)  
- Mountain brush-tailed possum (*T. cunninghami*) | NSW, Vic, SA, WA | Unknown |
<table>
<thead>
<tr>
<th><strong>Giardia sp.</strong></th>
<th><strong>Host (s)</strong></th>
<th><strong>Origin/Distribution</strong></th>
<th><strong>Clinical significance</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G. duodenalis</strong>&lt;br&gt;(Assemblage B)&lt;sup&gt;6,8&lt;/sup&gt;</td>
<td>Tammar wallaby&lt;br&gt;- Parma wallaby&lt;br&gt;- Quokka&lt;br&gt;- Red kangaroo&lt;br&gt;- Yellow-footed rock-wallaby&lt;br&gt;- Swamp wallaby&lt;br&gt;- Western grey kangaroo&lt;br&gt;- Brush-tailed rock-wallaby&lt;br&gt;- Tiger quoll (<em>Dasyurus maculatus</em>)</td>
<td>NSW, WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>G. duodenalis</strong>&lt;br&gt;(Assemblage C)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Southern brown bandicoot&lt;br&gt;- Bush rat (<em>Rattus fuscipes</em>)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>G. duodenalis</strong>&lt;br&gt;(Assemblage D)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Bush rat</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>G. duodenalis</strong>&lt;br&gt;(Assemblage E)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Ash-grey mouse (<em>Pseudomys albocinereus</em>)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>G. duodenalis</strong>&lt;br&gt;(Assemblage F)&lt;sup&gt;7&lt;/sup&gt;</td>
<td>Bush rat</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>G. peramelis</strong>&lt;sup&gt;4,7,9&lt;/sup&gt;</td>
<td>Southern brown bandicoot</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Giardia sp.</strong>&lt;br&gt;&lt;sup&gt;4,2&lt;/sup&gt;</td>
<td>Bennetts wallaby (<em>Macropus rufogriseus</em>)&lt;br&gt;- Common brush-tailed possum&lt;br&gt;- Common ringtail possum (<em>Pseudocheirus peregrinus</em>)&lt;br&gt;- Common wombat (<em>Vombatus ursinus</em>)&lt;br&gt;- Southern brown bandicoot&lt;br&gt;- Eastern barred bandicoot (<em>Perameles gunnii</em>)&lt;br&gt;- Tasmanian pademelons (<em>Thylogale billardieri</em>)&lt;br&gt;- Long-nosed potoroo</td>
<td>Tas.</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Giardia sp.</strong></td>
<td><strong>Host (s)</strong></td>
<td><strong>Origin/Distribution</strong></td>
<td><strong>Clinical significance</strong></td>
</tr>
<tr>
<td>----------------</td>
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<td>-------------------------</td>
</tr>
</tbody>
</table>
| *Giardia sp.*<sup>1,3</sup> | -Tasmanian devils (*Sarcophilus harrisii*)  
-Southern brown bandicoot  
-Bandicoots (*Perameles* spp.)  
-Common brush-tailed possum  
-Kangaroos (*Macropus* spp.) | NSW, Tas. | Unknown |

<sup>1</sup>Mackerras, 1958; <sup>2</sup>Bettiol, 1997; <sup>3</sup>Millstein & Goldsmith 1997; <sup>4</sup>Adams e al., 2004; <sup>5</sup>McCarthy et al., 2008; <sup>6</sup>Thompson et al., 2008; <sup>7</sup>Thompson et al., 2010; <sup>8</sup>Vermeulen et al., 2015; <sup>9</sup>Hillman et al., 2016  
*No molecular studies were conducted on these isolates.

### 1.7.1 Epidemiology

While wildlife is commonly infected with *Giardia*, little is known about the prevalence of this parasite in Australian native mammals. Previous studies have reported prevalences of 19% in Bennetts wallaby (*Macropus rufogriseus*), 16% in common brush-tailed possums, 20% in common wombats, 62% in bandicoots (*Isoodon obesulus, Perameles gunnii*), 23% in Tasmanian pademelons (*Thylogale billardierii*), 29% in long-nosed potoroos (*Perameles tridactylus*) and 4.1% in western grey kangaroos (Bettiol et al., 1997; McCarthy et al., 2008).

A molecular investigation reported a 13-14% prevalence of *G. duodenalis* (assemblages A and B) in captive and wild marsupials in WA, Vic and SA (Thompson et al., 2008). Another recent molecular survey undertaken throughout the mainland and some off-shore islands of WA revealed that the prevalence of *Giardia* in native mammal species from these areas was low, with only 4.8% (17/351) of the samples testing positive (Thompson et al., 2010).
1.7.2 Pathogenesis and clinical significance of giardiasis in Australian native mammals

*Giardia* is not invasive and lives and multiplies by asexual multiplication on the luminal surface of the small intestine of its vertebrate hosts. The pathogenesis of *Giardia* involves a multifactorial pathophysiological process that is both parasite and host dependent and is variable in its expression in terms of symptomatology, clinical consequences and severity (Certad et al., 2017). The symptoms of giardiasis include acute or chronic diarrhoea, dehydration, abdominal pain and weight loss (Thompson, 2000). It seems likely that these symptoms may vary depending upon the ‘strain’ of the parasite and nutritional factors and immune status of the host, as well as concurrent enteric infections with other parasites and infectious agents (Thompson, 2004).

One of the fundamental processes that are triggered by an infection with *Giardia* is altered epithelial permeability that appears to result from a direct cytopathic effect induced by products of the parasite (Buret et al., 2002). The resultant increased epithelial permeability leads to an inflammatory response and both digestive and absorptive changes (Scott et al., 2002). *Giardia* also induces apoptosis that correlates with the loss of epithelial barrier function the subsequent increase in permeability (Chin et al., 2002).

The impacts of enteric parasites on wildlife health are not yet well elucidated with infections with *G. peramelis* subclinical in quendas in WA (Adams et al., 2004; Hillman et al., 2016; Thompson & Ash, 2016).

1.7.3 Diagnosis

Diagnosis of *Giardia* by traditional microscopic methods following the application of faecal concentration techniques, remain a reliable indicator of infection. The development of direct immunofluorescence microscopy has generally improved the
sensitivity of detecting faecal *Giardia* cysts (O’Handley, 2002). However, the detection of *G. duodenalis* by conventional or fluorescent microscopy or faecal ELISA is of limited epidemiological value.

As with other protozoan parasites, PCR-based methods developed to detect *Giardia* have excellent sensitivity and specificity, and are more convenient than microscopy when a screening a large amount of samples (Morgan, 2000). Genetic markers that have been used for the detection of *Giardia* include: 18S rDNA, glutamate dehydrogenase (*gdh*), elongation factor 1- alpha (*ef1-a*) and triose phosphate isomerase (*tpi*) genes among others (Thompson, 2004).

**1.7.4 Treatment and control**

Nitroimidazoles and benzimidazoles are effective antigiardial drugs for treating infections in humans and domestic animals. However, even though treatment may be effective in eliminating infection, re-infection frequently occurs if the sources of environmental contamination are not eliminated and the frequency of transmission is high (Thompson, 2004). There are no specific treatment recommendations for giardiasis in Australian mammals.

**1.7.5 Zoonotic potential and One Health implications**

The majority of reported *Giardia* infections in Australian mammals are zoonotic assemblages and are considered to reflect accidental infections in naïve hosts (Thompson, 2004). This suggests ‘reverse zoonotic transmission’ (*zooanthroponosis*) is an important factor that must be considered in understanding the epidemiology of infections with *Giardia*, particularly in Australian native mammals (Thompson & Ash, 2016).
The public health risks associated with marsupials in water catchments harbouring zoonotic *Giardia* isolates require further investigation. Recent studies suggest that animals are unlikely, in most cases, to be the original contaminating source of *Giardia* in waterborne outbreaks, although aquatic wildlife are capable of amplifying zoonotic assemblages of *Giardia* that may contaminate water (Thompson, 2004).

1.8 Thesis aims and objectives

The overarching aim of this thesis was to provide baseline data on the molecular prevalence, genetic diversity and potential clinical impact of blood-borne and enteric protozoan parasites on a range of native mammal species (including marsupials, rodents and bats) across a diverse range of locations in Australia.

Specifically, this thesis aimed to:

1. Use morphological and molecular tools to identify, characterise and determine the prevalence of *Trypanosoma, Babesia, Theileria, Cryptosporidium* and *Giardia* in a range of Australian native mammals;
2. Investigate the potential clinical impact of infections with these parasites on the hosts studied;
3. Investigate the role of Australian native mammals as potential reservoirs for known zoonotic protozoan pathogens.
Chapter 2 General Materials and Methods

2.1 Sample collection

The group of animals examined in this cross-sectional study comprised wild mammals which were either live-trapped or presented to wildlife hospitals due to illness or injuries. A total of 465 blood samples, 167 faecal samples and 91 ticks were collected between December 2009 and February 2016 from mammals belonging to seven species: common brush-tailed possum, northern brown bandicoot, northern quoll, koala, brush-tailed rabbit-rat, little red flying fox and grey-headed flying fox (Fig. 2.1). The sampling was conducted in nine sampling sites across four states/territories in Australia (Fig. 2.2). Information on sample sizes, sources and origins are summarized in Table 2.1.

Sampling was undertaken according to standard capture, handling and collection procedures by experienced wildlife veterinarians or research team personnel. All trapped animals suitable for the study were anaesthetised using a portable inhalational anaesthetic machine delivering isoflurane (Delvet Isoflurane, Delvet Pty Ltd, Seven Hills, NSW, Australia) in 100% medical-grade oxygen. Hospitalized koalas were induced for anaesthesia with an intramuscular administration of alfaxalone (Alfaxan1 CD RTU, Jurox Australia) at a dose rate of 3 mg/kg. Anaesthesia was then maintained using a combination of 1.5% isofluorane and oxygen at 1.5 L/min delivered by either mask or endotracheal intubation.

Blood samples of approximately 0.5-1 ml were collected from suitable superficial veins such as the femoral vein in bandicoots, the lateral tail and medial saphenous veins in possums, the jugular vein in quolls, the ventral tail or femoral vein in brush-tailed rabbit-rats, and the cephalic vein in koalas and bats. Faecal samples (0.5-1g) were opportunistically collected, as available, during the capture of common brush-tailed
possums, northern brown bandicoots, northern quolls and brush-tailed rabbit-rats. Blood and faeces were stored at -20°C freezer until processed at Murdoch University.

Tick samples were removed from koala hosts at the Australia Zoo Wildlife Hospital (AZWH), Beerwah, Qld and also supplied by Endeavour Veterinary Ecology Pty Ltd, Toorbul, Qld (Table 2.1). The ticks were stored in 70% ethanol at room temperature after collection until DNA extraction.

Figure 2.1. Australian native mammal species sampled: (a) Common brush-tailed possum (Trichosurus vulpecula) (b) Northern brown bandicoot (Isoodon macrourus) (c) Northern quoll (Dasyurus hallucatus) (d) Brush-tailed rabbit-rat (Conilurus penicillatus) (e) Koala (Phascolarctos cinereus) (f) Little red flying fox (Pteropus scapulatus) (g) Grey-headed flying fox (Pteropus poliocephalus).
Figure 2.2. Map of Australia showing the study sampling sites’ approximate geographical location.

All aspects of sample collection were approved by the Murdoch University Animal Ethics Committee (permit numbers W2284/09, W2591/13 and W2636/14) and Charles Darwin University-NT Animal Ethics Committee (permit numbers A11027 and A13026). Additionally, a permit for access to biological resources under part 8A of the Environment Protection and Biodiversity Conservation Regulations (2000) was obtained for sample collection within Kakadu National Park-NT (permit number RK846).

2.2 Tick identification

Tick instars and species were microscopically examined with an Olympus SZ6 stereomicroscope (Olympus, Centre Valley, PA, USA), with a Schott KL 1500 LED light source (Schott AG, Mainz, Germany), and morphologically identified using standard taxonomic keys (Roberts, 1970; Barker & Walker, 2014). All ticks analysed in
the present study belonged to the family Ixodidae; of which 56 (61.5%) were identified as *Ixodes tasmani* and 35 (38.5%) as *I. holocyclus*. In total, 82 (93.8%) of ticks were females. Of these, 49 (59.8%) belonged to the species *I. tasmani* and 33 (40.2%) to the species *I. holocyclus*. Only 9 ticks (7 *I. tasmani* and 2 *I. holocyclus*), were males. No nymphs or larvae were observed.

**Table 2.1. Number of samples collected per host species and geographic location**

<table>
<thead>
<tr>
<th>Origin</th>
<th>Host species</th>
<th>Wild-caught /Hospitalized</th>
<th>№ of blood samples</th>
<th>№ of faecal samples</th>
<th>№ of ectoparasites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kakadu National Park (NT)</td>
<td>- Common brush-tailed possum (<em>Trichosurus vulpecula</em>)&lt;br&gt;- Northern brown bandicoot (<em>Isoodon macrourus</em>)&lt;br&gt;- Northern quoll (<em>Dasyurus hallucatus</em>)</td>
<td>Wild-caught</td>
<td>8</td>
<td>6</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Coburg Peninsula (NT)</td>
<td>- Common brush-tailed possum&lt;br&gt;- Northern brown bandicoot&lt;br&gt;- Brush-tailed rabbit-rat (<em>Conilurus penicillatus</em>)</td>
<td>Wild-caught</td>
<td>54</td>
<td>44</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Bathurst Island (NT)</td>
<td>- Common brush-tailed possum&lt;br&gt;- Northern brown bandicoot&lt;br&gt;- Brush-tailed rabbit-rat</td>
<td>Wild-caught</td>
<td>26</td>
<td>43</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26</td>
<td>10</td>
<td>N/A</td>
</tr>
<tr>
<td>Groote Eylandt (NT)</td>
<td>- Northern brown bandicoot&lt;br&gt;- Northern quoll</td>
<td>Wild-caught</td>
<td>13</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peri urban areas of Darwin (NT)</td>
<td>- Common brush-tailed possum&lt;br&gt;- Northern brown bandicoot</td>
<td>Wild-caught</td>
<td>9</td>
<td>12</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Origin</td>
<td>Host species</td>
<td>Wild-caught/Hospitalized</td>
<td>N° of blood samples</td>
<td>N° of faecal samples</td>
<td>N° of ectoparasites</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>-------------------------------</td>
<td>-------------------------</td>
<td>---------------------</td>
<td>----------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Australian Zoo Wildlife Hospital (Qld)*</td>
<td>Koala (Phascolarctos cinereus)</td>
<td>Hospitalized</td>
<td>161</td>
<td>N/A</td>
<td>81</td>
</tr>
<tr>
<td>Port Macquarie Koala Hospital (NSW)*</td>
<td>Koala</td>
<td>Hospitalized</td>
<td>7</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Endeavour Veterinary Ecology Pty Ltd (Qld)</td>
<td>Koala</td>
<td>Wild-caught</td>
<td>N/A</td>
<td>N/A</td>
<td>10</td>
</tr>
<tr>
<td>Redcliffe, south-eastern Qld</td>
<td>Little red flying fox (Pteropus scapulatus)</td>
<td>Hospitalized</td>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Botanic Park, Adelaide (SA)</td>
<td>Grey-headed flying fox (Pteropus poliocephalus)</td>
<td>Wild-caught</td>
<td>87</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*Most of the wild koalas sampled at the Australia Zoo Wildlife Hospital (AZWH), Beerwah, Qld and the Koala Hospital, Port Macquarie, NSW originated from areas in south-east Qld or northern NSW. N/A=Not available

2.3 Morphological detection and characterisation of blood-borne protozoan parasites

2.3.1 Preparation of blood films

Thin blood smears were made from a drop of peripheral blood, air-dried and then fixed with methanol within four hours of sample collection. In the laboratory at Murdoch University, the smears were stained with either Diff Quick (Siemens, Germany) or Wright-Giemsa (Hematek® Stain Pak) and then coverslip mounted using DePeX mounting medium Gurr (Merck Pty. Limited, Kilsyth, Vic, Australia).
2.3.2 Light microscopy

Stained blood films were systematically examined by light microscopy at 200× and 400× magnification for the presence of protozoan parasites, using a BX50 microscope (Olympus, Japan) with screen views generated by a DP Controller (version 3.2.1.276, Olympus, Japan). Digital light micrograph images of any haemoprotozoans detected were taken at 1000 x magnification using Image-Pro Express software (Media Cybernetics, Inc., Bethesda, Maryland, USA).

2.3.3 Morphometric analysis

Digital images of the organisms identified in the blood films were imported into software Image J (Abramoff et al., 2004) for morphometric analysis. Key morphological features of trypanosomes, such as total length (length of body measured along the mid-line including free flagellum), width (maximum width measured at the level of the nucleus including undulating membrane), PK (distance between the posterior end and the kinetoplast), KN (distance between the kinetoplast and posterior edge of the nucleus), NA (distance between the anterior edge of the nucleus and the anterior end of the body) and FF (length of the free flagellum) were measured according to parameters described by Hoare (1972) and Mackerras (1959) (Fig. 2.3). Means and standard errors were calculated and compared with available morphological measurements from previous studies.
2.4 Molecular detection and characterisation of blood-borne and enteric protozoan parasites

2.4.1 DNA extraction

Genomic DNA was isolated from 200 µl of whole blood using a MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies, Madison, Wisconsin, USA), according to the manufacturer’s instructions. DNA was eluted in 35 µl of TE buffer and stored at -20°C until required.

A Power Soil DNA Kit (MolBio, Carlsbad, California) was used to extract the DNA from 250 mg of each faecal sample. The DNA samples were stored frozen (-20 °C) until processed.

Prior to DNA isolation, ticks were surface sterilised in 10% sodium hypochlorite, washed in 80% ethanol and DNA-free PBS, and air-dried. Individual ticks were then cut into quarters with a sterile scalpel, placed in a 2 ml microtube with a 5 mm steel bead,
snap frozen in liquid nitrogen for 1 min and homogenized by shaking at 40 Hz for 1 min. Genomic DNA was isolated from tick homogenates using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations.

Extraction reagent blank controls (no sample added) were included alongside all DNA extractions.

2.4.2 DNA amplification of the 18S rRNA, GAPDH and gdh genes

Studies presented in this thesis utilised multiple amplification techniques and genetic markers to gain a broad picture of protozoan parasites’ genetic diversity in Australian native mammal species.

Overall, DNA isolated from blood samples was screened by PCR at the 18S rRNA gene for trypanosomes, piroplasms and haemogregarines. Amplification of Trypanosoma glycosomal glyceraldehyde phosphate dehydrogenase (GAPDH) gene was also conducted when additional genetic information was required for the characterisation of novel species. Tick samples were tested for the presence of Trypanosoma spp. using universal 18S rRNA primers.

DNA extracted from faecal samples was screened by PCR for Cryptosporidium at the 18S rRNA locus and also for Giardia spp. at the gdh locus and 18S rRNA loci.

General information on all PCR methods performed in this thesis and corresponding references are presented in Table 2.2. Specific details (e.g. reagents’ concentrations and thermocycling conditions) related to novel PCR approaches developed in the present study are provided within the relevant chapters (Chapters 4 and 5).
<table>
<thead>
<tr>
<th>Targeted parasite</th>
<th>Genetic marker</th>
<th>Molecular methods</th>
<th>Primers</th>
<th>Approximate product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trypanosoma</em></td>
<td>18S rRNA gene</td>
<td>Nested PCR and Sanger sequencing</td>
<td>External: SLF/S762R, Internal: S823F/S662R</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trypanosoma</em></td>
<td>18S rRNA gene</td>
<td>Nested PCR and Sanger sequencing</td>
<td>External: TRY927F, TRY927R, Internal: SSU561F/ SSU561R</td>
<td>927</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trypanosoma</em></td>
<td>18S rRNA gene</td>
<td>Single-round PCR and Sanger sequencing</td>
<td>S825F/S662R</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trypanosoma</em></td>
<td>18S rRNA gene</td>
<td>Hemi-nested PCR followed by NGS</td>
<td>External: S825F/ TryAll R1 (5’-GACTGTAACCTCAAAGCTTTGC-3’)*, Internal: S825F/S662R</td>
<td>350</td>
</tr>
<tr>
<td></td>
<td>GAPDH gene</td>
<td>Hemi-nested PCR and Sanger sequencing</td>
<td>External: GAPDHF/GAPDHR, Internal: GAPDHF/G4a</td>
<td>800</td>
</tr>
<tr>
<td><em>Trypanosoma</em></td>
<td>18S rRNA gene</td>
<td>Nested PCR and Sanger sequencing</td>
<td>External: S823F/ S662R, Internal: TVIF/TVIR</td>
<td>350</td>
</tr>
<tr>
<td><em>Trypanosoma</em></td>
<td>18S rRNA gene</td>
<td>Nested PCR and Sanger sequencing</td>
<td>External: TVEF/TVER, Internal: TVIF/TVIR</td>
<td>350</td>
</tr>
<tr>
<td><em>Babesia/Theileria</em></td>
<td>18S rRNA gene</td>
<td>Nested PCR and Sanger sequencing</td>
<td>External: BTF1/BTR1, Internal: BTF2/BTR2</td>
<td>850</td>
</tr>
<tr>
<td>Targeted parasite</td>
<td>Genetic marker</td>
<td>Molecular methods</td>
<td>Primers</td>
<td>Approximate product length (bp)</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------------</td>
<td>------------------</td>
<td>---------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Haemogregarines</td>
<td>18S rRNA gene</td>
<td>Single-round PCR and Sanger sequencing</td>
<td>HEMO1/HEMO2</td>
<td>900</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>18S rRNA gene</td>
<td>Nested PCR and Sanger sequencing</td>
<td>External: 18SiCF2/18SiCR2 Internal: 18SiCF1/18SiCR1</td>
<td>400</td>
</tr>
<tr>
<td>Giardia</td>
<td>gdh locus</td>
<td>Nested PCR and Sanger sequencing</td>
<td>External: GDHeF/GDH2 Internal: GDHiF/GDH4</td>
<td>800</td>
</tr>
<tr>
<td>Giardia</td>
<td>18S rRNA gene</td>
<td>Nested PCR and Sanger sequencing</td>
<td>External: RH11/RH4LM Internal: GiAR18SeR/GiAR18SiR</td>
<td>130</td>
</tr>
</tbody>
</table>

1 Maslov et al., 1996; 2 Hopkins et al., 1997; 3 Noyes et al., 1999; 4 Perkins and Keller, 2001; 5 Ryan et al., 2003; 6 Hamilton et al., 2004; 7 Read et al., 2004; 8 Jefferies et al., 2007; 9 Caccio et al., 2008; 10 McInnes et al., 2009; 11 Botero et al., 2013; 12 Hijjawi et al., 2016; 13 Wicks et al., 2016. *Primer and PCRs designed during the present study

2.4.3 PCR controls, agarose gel electrophoresis and purification of PCR products

The PCRs were performed in 25µl reaction volumes and then run on a 2% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA). Gel bands were visualized with a dark reader trans-illuminator (Clare Chemical Research, USA) and those corresponding to the expected length were excised and purified using an in-house filter tip method as described (Yang et al., 2013). DNA extraction blanks, negative controls (no sample added) and positive controls were included in each PCR batch.

DNA extraction and PCR setup were all performed in separate physically contained exclusion hoods, and post-PCR procedures were performed in a separate dedicated laboratory.
2.4.4 Sequencing of the 18S rRNA, GAPDH and *gdh* genes

Sanger sequencing of the purified PCR products was performed using the corresponding internal primers diluted at 3.2 picomoles with an ABI Prism™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA Analyser.

NGS was conducted in one particular study within this thesis, thus the method is described in the relevant chapter.

2.5 Phylogenetic analyses

Phylogenetic analyses were conducted for each group of parasites which produced clean partial nucleotide sequences at the 18S rRNA, GAPDH and/or *gdh* loci. To avoid duplicates, the sequences belonging to the same taxa were firstly aligned with each other by MUSCLE (Edgar, 2004) using the software in MEGA 6 (Tamura et al., 2013). In cases where two or more sequences were 100% identical, and sourced from the same host species, the longest one containing the least number of ambiguous bases was selected as the group representative isolate for evolutionary inferences. For each phylogenetic analysis, DNA sequences were imported into Geneious R7 (Kearse et al., 2012) or MEGA 6 and combined with additional sequences retrieved from GenBank. Accession numbers for all sequences included in the analyses are provided on the left of each isolate name in the phylogenetic trees. The sequences were aligned by MUSCLE or CLUSTAL W (Larkin et al., 2007) and, after global-trimming, the most appropriate nucleotide substitution model (i.e. with the lowest Bayesian information criterion - BIC score) was selected using the dedicated function (Find best DNA/protein model) in MEGA 6.
The analyses of each dataset were performed using both neighbour-joining (Saitou & Nei, 1987) and maximum likelihood (Tamura et al., 2011) methods. Each chapter discusses the specific nucleotide substitution model utilised to infer evolutionary relationships among each group of parasites identified. All phylogenetic trees were drawn to scale using the resources of MEGA 6, with branch lengths measured in the number of substitutions per site. The bootstrapping method (n = 500 replicates) was used to infer reliability for internal branch and the percentage (> 60%) of trees, in which the associated taxa clustered together is shown next to the branches. Estimates of genetic divergence between sequences were also generated in MEGA 6, based on the same model used to infer the phylogenetic relationships.

2.6 Analysis of the clinical impact of blood-borne and enteric protozoan parasites

Assessment of clinical parameters of the animals examined and correlation of clinical data with microscopy and molecular results were conducted during two studies presented in this thesis. The approaches utilised to obtain clinical, haematological, biochemical and pathological data are discussed within the respective chapters.

2.7 Statistical analyses

Prevalences (%) were calculated as no positives/ no samples x 100. Confidence intervals (95%) were calculated in Microsoft Excel version 2013 using the exact binomial method. Statistical analyses of morphometric parameters (when applicable) were conducted using the one sample t-test, in the software PAST 1.43 (Hammer et al., 2001).
Chapter 3 Prevalence, genetic diversity and potential clinical impact of blood-borne and enteric protozoan parasites in native mammals from Northern Australia

3.1 Preface

This chapter is a modified version of the article:

3.2 Introduction

Evidence from biodiversity surveys across the tropical north or “Top End” of the NT in Australia strongly suggests that many small to medium sized mammal species are in rapid and broad-scale decline, even in protected areas such as Kakadu National Park (Woinarski et al., 2010; Woinarski et al., 2011; Ziembicki et al., 2015). The declines in both diversity and abundance have probably been occurring for over 20 years, and infectious disease may be a potential contributor, acting in synergy with other factors such as introduced predators and changed fire regimes (Woinarski et al., 2015; Ziembicki et al., 2015).

The present study was part of a broader two-year collaborative project, conducted in the NT, to investigate the potential role of disease in the decline of native mammal species (Reiss et al., 2015). It was specifically aimed at identifying and characterising blood-borne and enteric protozoan parasites in these species using morphological and molecular analyses. This research also sought to investigate the potential clinical impact these protozoan parasitic infections may have upon their hosts.

3.3 Materials and Methods

3.3.1 Sampling

The sampling was conducted from June 2013 to December 2014, across five different locations within the NT (Fig 3.1): peri-urban areas around Darwin, Kakadu National Park, Bathurst Island, Groote Eylandt and Cobourg Peninsula (for simplicity, these locations will be referred to as Darwin, Kakadu, Bathurst, Groote and Cobourg hereafter). These sites were pre-selected areas which formed parts of mammal survey programs conducted by the Department of Environment and Natural Resources (DENR) in the NT. Sampling areas in Darwin were selected by DENR as representative of relatively disturbed environments proximal to human settlement, whereas Cobourg,
Bathurst and Groote represented relatively remote and less disturbed areas. Kakadu has intermediate proximity to human settlements and is relatively more disturbed than the islands, due to the presence of feral herbivores and pigs, a pre-park history of grazing, and significantly altered fire regimes. Kakadu is also where north Australian mammal declines have been most documented (Woinarski et al., 2010). Small cage and Elliot traps were used at sampling sites, in a variety of layouts depending on location, however most commonly in either a line of traps arranged as a grid, or a 50m by 50m square with 16 Elliot and 8 small cage traps distributed evenly. Traps were open for 3-5 nights, and cleared at dawn each morning.

Figure 3.1. Geographic representation of survey areas within the NT and location of the territory in the Australian continent (insert).
A total of 209 blood and 167 faecal samples were collected from animals belonging to four target mammal species: northern brown bandicoot, common brushtail possum, northern quoll and brush-tailed rabbit-rat. These species were selected as they are representatives of each major taxonomic group that have undergone decline in the NT: Phalangeridae (possums), Peramelidae (bandicoots), Dasyuridae (carnivorous marsupials) and Muridae (rodents). In addition, logistics dictated a focus on species of relatively large body size that were most likely to be trapped in reasonable numbers, to acquire sufficient specimen amounts and sample size. The numbers of samples collected from each target species across the five sampling sites are included in Table 2.1.

Anaesthesia and sample collection from trapped animals suitable for the study were conducted as described in chapter 2 (section 2.1).

3.3.2 Clinical assessment

During the fieldwork, all animals were submitted to a full physical examination conducted by an experienced wildlife veterinarian or veterinary nurse. A range of standard data and assessments such as species, sex, age, and body and health condition were recorded.

Age class was assessed subjectively as adult, sub-adult or juvenile, based on the individual’s body size, morphometric measurements and evidence or otherwise of reproductive activity. Molar tooth wear was assessed subjectively in all species other than rodents, and classified on a 4 point scale (nil, mild, moderate or significant wear) to assist in ageing of adults.

Health condition was also assessed subjectively as either normal or abnormal, based on a range of criteria including demeanour (quiet, agitated, highly agitated), state of
alertness (unresponsive, reduced response, responsive), condition of the skin and pelage, assessed body condition and the presence of obvious wounds or disease. Abnormal health status was further classified as mild, moderate or severe and acute or chronic in nature.

3.3.3 **Haematology and biochemistry**

Blood samples were sent to laboratories in Darwin (BVL) and Adelaide (Gribbles Veterinary Laboratory, Wayville SA, Australia) for full haematological analyses, except packed cell volume (PCV) and total plasma protein (TPP) readings, which were performed in the field using a microcentrifuge and a hand-held refractometer, within 6 hours of sample collection. In the laboratories, total plasma protein (TPP), total red cell count (TRCC), total white cell count (TWCC), red blood distribution, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin content (MCHC) and platelet count were determined.

Serum biochemical parameters were determined on an automated analyser (Konelab 20, Thermo Electron, Vica, Australia). The following variables were measured: sodium, potassium, chloride, urea, creatinine, glucose, total bilirubin, aspartate amino transferase (AST), alanine transaminase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), globulin (Glob), albumin (Alb), albumin/globulin ratio, calcium, phosphate, magnesium, iron and creatine kinase (CK). Both haematological and biochemical tests conducted in the laboratories were performed within 72 hours of sample collection.

3.3.4 **Microscopy and morphometric analysis**

Thin blood smears were prepared, stained and examined by light microscopy according to the methods described in chapter 2 (sections 2.3.1 and 2.3.2).
Digital light micrograph images of any haemoprotozoans detected were taken at 1,000 x magnification and then used for morphometric analysis (see section 2.3.3). Morphological features were compared with previously reported morphological measurements of corresponding haemoprotozoans infecting Australian wild mammals.

3.3.5 Molecular analyses

DNA extractions, PCR assays and purification of PCR products were conducted as described in chapter 2 (sections 2.4.1 to 2.4.3). Initially, five genera of potential protozoan pathogens to Australian wildlife were targeted in the molecular investigation: *Trypanosoma*, *Babesia*, *Theileria*, *Cryptosporidium* and *Giardia*. DNA samples extracted from blood (see section 2.4.1) were screened for *Trypanosoma* at the 18S rRNA locus using a nested set of trypanosome-universal primers (SLF/S762R and S823F/S662R) as previously described (Maslov et al., 1996; McInnes et al., 2009). Universal piroplasm primers were used to amplify an approximately 850 bp fragment of the 18S rRNA gene, in a nested PCR performed as described by Jefferies et al. (2007). During the present study, the piroplasm primers also amplified nucleotide sequences from the haemogregarine *Hepatozoon*. However, these sequences did not align with previously reported *Hepatozoon* isolates from quendas, due to different primer sets used and thereby amplification of divergent regions of the 18S rRNA. Therefore, a PCR assay specific for haemogregarines was used to produce new sequences suitable for a complete phylogenetic analysis comprising all relevant species. An approximately 900 bp fragment of the *Hepatozoon* 18S rDNA was amplified using a single round haemogregarine specific PCR, as previously described (Perkins & Keller, 2001; Wicks et al., 2006).

DNA extracted from faecal samples (see section 2.4.1) was screened by nested PCR for *Cryptosporidium* 18S rDNA as previously described (Ryan et al., 2003). The samples
were also tested by nested PCR for *Giardia* spp. at the *gdh* locus, using external and internal forward primers (GDHeF and GDHiF) designed by Read et al. (2004) and external and internal reverse primers (GDH2 and GDH4) sourced from Caccio et al. (2008), in an assay as previously described (Hijjawi et al., 2016). *Giardia*-positive samples at the *gdh* locus were also screened at the 18S rRNA gene using the external primers RH11/RH4LM and internal primers GiAR18SeR/GiAR18SiR as described by Hopkins et al. (1997) and Read et al. (2004).

Sanger sequencing was performed in all purified PCR products as described in chapter 2 (section 2.4.4). Nucleotide sequences obtained in this investigation were deposited in GenBank under the accession numbers KX361174-84 and KX369229-32.

### 3.3.6 Phylogenetic analysis

Phylogenetic analyses and estimation of genetic divergence between sequences were conducted for each group of parasites which produced clean partial nucleotide sequences at the 18S rRNA and/or *gdh* loci, as described in chapter 2 (section 2.5). The phylogenetic relationships of new trypanosome isolates with other *Trypanosoma* spp. were inferred using the Kimura 2-parameter model (Kimura, 1980). For the other parasites (*Babesia* spp., *Hepatozoon* spp., *Cryptosporidium* spp. and *Giardia* spp.), the phylogenetic relationships were inferred based on the Tamura 3-parameter model (Tamura, 1992). All positions with less than 95% site coverage were removed.

### 3.3.7 Statistical analysis

Prevalences (%) were calculated as described in chapter 2 (section 2.7). Univariate analyses of the clinical, haematological and biochemical data were performed in “R” version 3.0.2 (R Core Development Team, 2013), where the dependent variables were the presence or absence of protozoan parasites. Measures of association were examined
for nominal data (location, species, sex, season) and ordinal data (age class, body condition, tooth wear, ectoparasite burden) using the Odds Ratio (OR) with 95% CI, and p-values using the chi-square test (all categories sample sizes > 5) or Fisher’s exact test (any category sample size < 5), with significance set at the level of 0.05.

Continuous data such as PCV, TPP, Alb, Glob, TWCC and body weight were checked for normality using the Shapiro-Wilk test and grouped by infecting parasite genus. Differences for normal data (all variables) were tested for significance using ANOVA. All measures of association and difference were analysed by location, species, and then location and species, where samples sizes permitted. Continuous data could only be evaluated within species (and sexes or age classes) to account for differences between these groups.

3.4 Results

3.4.1 Prevalence and molecular characterization

3.4.1.1 Blood-borne protozoan parasites

Overall, 22.5% (95% CI: 17.0-28.8%) of the animals tested were positive for haemoproteozoa. Molecular analysis based on a trypanosome universal assay revealed the presence of *Trypanosoma* spp. in 39 samples (18.6%; 95% CI: 13.6-24.6%), whereas *Babesia* and *Hepatozoon* were detected in 11 samples each (5.3%; 95% CI: 2.7-9.2%). The prevalence of *Hepatozoon* was also confirmed by a haemogregarine-specific PCR. Overall prevalence estimates of *Trypanosoma*, *Babesia*, and *Hepatozoon* are represented in Fig 3.2 (A).

The prevalence of each group of blood-borne protozoans across different host species is shown in Fig 3.2 (B). The presence of trypanosomes was confirmed in nine out of 38 possums (23.7%; 95% CI: 11.4-40.2%) and in 30 out of 113 bandicoots (26.6%; 95%
CI: 18.7-35.7%); while Babesia and Hepatozoon were found in bandicoots only, at a prevalence of 9.7% (95% CI: 2.7-9.2%) each. PCR and sequence analyses also revealed that 5% (95% CI: 1.8-10.8%) of the bandicoots were co-infected with Trypanosoma. and Hepatozoon whilst 7% (95% CI: 3.1-13.4%) were co-infected with Trypanosoma and Babesia. Northern quolls and brush-tailed rabbit-rats were negative for blood-borne protozoan parasites (95% CI: 0-7% and 0-41%, respectively).

Prevalence estimates (with 95% CI) of Trypanosoma, Babesia and Hepatozoon were also calculated across the five geographical sampling sites (Fig 3.2-C). Trypanosomes were found in animals from all sites and the prevalence was significantly higher in Darwin (68.8%; 95% CI: 45.5-86.9%) and Cobourg (34.1%; 95% CI: 22.5-47.2%), compared to Bathurst (8.4%; 95% CI: 2.6-19.3%), Kakadu (4.6%; 95% CI: 0.2-21.8%) and Groote (4.5%; 95% CI: 0.6-14.6%). This site difference remained when examining the effect within bandicoots only, where the odds of trypanosome infection was significantly higher in bandicoots from Darwin (OR = 13.7, 95% CI: 3.1-59.7, p = 0.0004) and Cobour (OR = 2.9, 95% CI: 1.0-8.1, p = 0.04) compared to all other sites. Bandicoots positive for Babesia were from three different locations (Darwin, Cobour and Bathurst), whereas Hepatozoon was identified in bandicoots from all locations except Groote. The differences between prevalence of Babesia and Hepatozoon across locations were not statistically significant.
Figure 3.2. Prevalence of *Trypanosoma*, *Babesia* and *Hepatozoon* in native mammal species from the NT, by PCR. (A) Overall prevalence. (B) Prevalence per host species (C) Prevalence across surveyed locations. Sample sizes are indicated in the figure legends. Error bars represent 95% confidence interval. Within each graph (A, B and C), values represented by different letters are statistically distinct (p < 0.05).
3.4.1.2 Enteric protozoan parasites

Molecular investigation revealed that five out of 167 (3.0%; 95% CI: 1.0-6.8%) faecal DNA samples were positive for enteric protozoan parasites. PCR analysis at the 18S rRNA gene confirmed the presence of Cryptosporidium in one individual only (0.6%; 95% CI: 0-3.3%), while four faecal DNA samples (2.4%; 95% CI: 0.7-6.0%) were positive for Giardia at the gdh locus. The samples positive for Giardia DNA at the gdh locus were also tested at the 18SrRNA locus; this confirmed the species to be G. peramelis, as previously described in quendas (Hillman et al., 2016).

Table 3.1 shows all enteric protozoan species detected in the present investigation across different host species and locations. Prevalence estimates of G. peramelis per host species are presented in Table 3.2.

Table 3.1. Enteric protozoan parasites isolated from three native mammal species from the Northern Territory of Australia

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Parasite species</th>
<th>Host species</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTF 20</td>
<td><em>Giardia peramelis</em></td>
<td>Northern-brown bandicoot</td>
<td>Bathurst</td>
</tr>
<tr>
<td>NTF 26</td>
<td><em>Cryptosporidium</em> brush-tail possum genotype</td>
<td>Brush-tail possum</td>
<td>Bathurst</td>
</tr>
<tr>
<td>NTF 132</td>
<td><em>Giardia peramelis</em></td>
<td>Brush-tail rabbit-rat</td>
<td>Darwin</td>
</tr>
<tr>
<td>NTF 136</td>
<td><em>Giardia peramelis</em></td>
<td>Brush-tail possum</td>
<td>Darwin</td>
</tr>
<tr>
<td>NTF 137</td>
<td><em>Giardia peramelis</em></td>
<td>Brush-tail possum</td>
<td>Darwin</td>
</tr>
</tbody>
</table>
Table 3.2. Prevalence of *Giardia peramelis* in native mammals from northern Australia

<table>
<thead>
<tr>
<th>Host species</th>
<th>Nº positives/ tested</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern brown bandicoot</td>
<td>1/62</td>
<td>1.6</td>
<td>0-8.7</td>
</tr>
<tr>
<td>Common brushtail possum</td>
<td>2/58</td>
<td>3.4</td>
<td>0.4-11.9</td>
</tr>
<tr>
<td>Northern quoll</td>
<td>0/10</td>
<td>0</td>
<td>0-30.8</td>
</tr>
<tr>
<td>Brush-tail rabbit-rat</td>
<td>1/37</td>
<td>2.7</td>
<td>0.1-14.2</td>
</tr>
<tr>
<td>Total</td>
<td>4/167</td>
<td>2.4</td>
<td>0.7-6.0</td>
</tr>
</tbody>
</table>

### 3.4.2 Phylogenetic analysis

#### 3.4.2.1 Blood-borne protozoan parasites

Phylogenetic analyses of trypanosomes using both neighbour-joining and maximum likelihood methods produced concordant tree topologies. The phylogenetic tree based on the neighbour-joining approach is presented in Fig. 3.3. All trypanosome sequences sourced from bandicoots clustered within the *T. vegrandis* clade, of which 24 were 100% identical to each other (represented in the tree as *T. vegrandis* NTB42) and also identical to the previously described *T. vegrandis* genotype 6 (G6) (Fig. 3.3). Another group of five identical sequences, represented by *T. vegrandis* NTB72, also clustered with *T. vegrandis* G6, exhibiting 99% genetic similarity to this isolate (one single nucleotide polymorphism - SNP). Although the phylogenetic position of the remaining isolate (NTB84) within a broader clade comprising *T. copemani, T. gilletti* and *T. vegrandis* was notable (bootstrap value = 97%), its association with the *T. vegrandis* genotypes was poorly supported (bootstrap values < 60%). In addition, the isolate exhibited 10 SNPs and a relatively larger genetic
distance (7%) from its most closely related genotype (T. ve grandis genotype 3) at the 18S rRNA gene, suggesting it is a unique genotype (Fig. 3.3).

Phylogenetic analysis also revealed that Trypanosoma 18S rDNA partial sequences identified in possums grouped within the T. noyesi clade, which is closely related to the T. cruzi clade of trypanosomes and comprises isolates sourced from a range of Australian marsupial species. Nine sequences identical to each other and represented by the isolate NTB15 were identical to T. noyesi H25 and T. noyesi AP-2011a-64 in this analysis. The remaining isolate NTB58 exhibited four SNPs and a small genetic divergence (2%) from NTB15, T. noyesi H25 and T. noyesi AP-2011a-64.
Figure 3.3. Phylogenetic relationships of new reported marsupial-derived trypanosome isolates with other *Trypanosoma* spp., based on 18S rDNA partial sequences (~350bp). Evolutionary history was inferred using the neighbour-joining method, based on the Kimura 2-parameter method (Kimura, 1980). Bootstrap values (> 60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site.
Concordant phylogenetic trees were obtained for Babesia and Hepatozoon sequences by neighbour-joining (data not shown) and maximum likelihood (Figs. 3.4 and 3.5). The partial 18S rDNA sequences identified as Babesia (n = 11) were identical to each other and associated with the B. macropus clade (Fig. 3.4). The new reported sequences were genetically distinct from all previously reported isolates and exhibited 99% similarity to B. macropus from eastern grey kangaroos (eight nucleotide differences), 98% similarity to a Babesia sp. sourced from a woylie or brush-tailed bettong (15 nucleotide differences) and 97% similarity to a wallaby-derived isolate characterised as B. macropus (21 nucleotide differences). In the present analysis, the kangaroo-derived B. macropus diverged 1.2% from its woylie-derived sister species and 2% from wallaby-derived B. macropus.

During preliminary analysis, the Hepatozoon sequences obtained using piroplasm primers did not align with previously reported sequences sourced from Western Australian marsupials (data not shown). Hence, another set of primers was used to obtain new Hepatozoon 18S rDNA sequences, which were then compared to a more representative group of Hepatozoon spp. Phylogenetic reconstructions revealed four distinct isolates, either identical or very closely related to previously reported Hepatozoon sp. from quendas from WA (Fig. 3.5). A group of eight sequences identical to each other and represented by Hepatozoon sp. NT72 in the tree, shared 100% identity with Hepatozoon sp. isolates B3, B11 and B12, while sequences NT30 and NT59 showed high similarity to the same three southern brown bandicoot-derived isolates (two SNPs; genetic distance = 0.27%). The Hepatozoon sp. NT31 also clustered within the “Australian marsupials” clade and was most closely related to Hepatozoon sp. NT30 (three SNPs; genetic distance = 0.4%) and Hepatozoon sp. B27 (five SNPs; genetic distance = 0.7%).
Figure 3.4. Phylogenetic analysis of *Babesia* spp. with the bandicoot-derived *Babesia* sp., based on 18S rDNA partial sequences (~650bp). Evolutionary history was inferred using the maximum likelihood method, based on the Tamura 3-parameter model (Tamura, 1992). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site.
Figure 3.5. Phylogenetic relationships of new reported bandicoot-derived isolates of *Hepatozoon* sp. with other *Hepatozoon* spp., based on 18S rDNA partial sequences (~800bp). Evolutionary history was inferred using the maximum likelihood method based on the Tamura 3-parameter model (Tamura, 1992). Bootstrap values (> 60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site.

3.4.2.2 Enteric protozoan parasites

Phylogenetic analysis using both neighbour-joining and maximum likelihood methods revealed that the *Cryptosporidium* sp. isolate from a possum from Bathurst was 99% similar (one SNP) to *Cryptosporidium* sp. isolate BTP1, previously reported in common brush-tailed possums from New South Wales (NSW) (Hill et al., 2008) (data not shown).
Figure 3.6. Phylogenetic analysis of *G. peramelis* and *G. duodenalis* assemblages, based on *gdh* partial sequences (~400 bp). Evolutionary history was inferred using the maximum likelihood method, based on the Tamura 3-parameter model (Tamura, 1992). Bootstrap support (> 60%) is indicated at the left of each node. The scale bar is the proportion of base substitutions per site.

Four partial *Giardia* DNA sequences were amplified at the *gdh* locus. Of these, two sequences sourced from possums (NTF 136 and NTF 137) showed high similarity to each other (2 SNP’s). For this reason and the relatively short length of isolate NTF137 (340 bp), this isolate was removed from the final phylogeny dataset. Phylogenetic reconstructions placed the sequences in a separate and strongly bootstrap supported clade (100%), basal to all *G. duodenalis* assemblages (maximum likelihood analysis is presented in Fig. 3.6). A distance similarity matrix confirmed the new sequences to be distinct from all assemblages within the *G. duodenalis* species cluster, with a minimum genetic distance of 12% from *G. duodenalis* assemblage C (from a dog). Unfortunately, *gdh* sequences from the recently described *G. peramelis* from quendas (Hillman et al., 2016) were
not available in GenBank. Therefore, partial 18S rRNA gene sequences were generated and phylogenetic reconstructions at this locus revealed that the newly reported *Giardia* from northern Australian mammals grouped with the unique *G. peramelis* in a well-supported clade (bootstrap value = 82%) (data not shown) and exhibited 1% genetic distance from *G. peramelis*. This finding suggests the isolates belong to the same species, as in this analysis the minimum genetic distance between two distinct species was 4% (between *G. microti* and *G. duodenalis* assemblage D). At the 18S rRNA locus, the *G. peramelis* isolates were 85% similar to their most closely related species (*G. duodenalis* assemblage C).

### 3.4.3 Microscopy and morphometric analysis

Light microscopy confirmed the presence of *Hepatozoon* sp. in two out of 11 animals positive for this parasite by PCR (Fig. 3.7). The gamonts (final development stage within the vertebrate host) observed were oval, relatively large, and easily detected by microscopy analysis of Giemsa stained blood films. Both intra and extra-erythrocytic forms were observed, all of them exhibiting a sub-terminal granular nucleus, slightly narrower than the gamonts’ width.
Eleven organisms were measured in total. The parasites’ lengths ranged from 7.5 – 9.3 µm (mean = 8.47 µm ± 0.15) and the observed range for width was 3.02 – 4.54 µm (mean = 3.61 µm ± 0.11).

The organisms were visually detected in blood films from bandicoots (NT2 and NT30), which provided *Hepatozoon* DNA sequences identical to each other and 99.6% similar to *Hepatozoon* sp. isolates B8, B13, B27 and B28 from Western Australian bandicoots. All blood films analysed were negative for other blood-borne protozoan parasites.

### 3.4.4 Potential clinical impact of protozoan parasites

Of individual animals examined during sample collection, the majority (91.8%) were assessed to be in good health and body condition. There were no obvious clinical associations between the presence or absence of protozoan parasites of any type and the clinically assessed health status of the host ($p > 0.05$).

Bandicoots positive for trypanosomes had significantly lower PCV ($p = 0.046$) than negative bandicoots. Animals with lower tooth wear scores (0, 1) were more likely to be trypanosome positive.
positive than those with higher tooth wear scores (2, 3) (OR = 2.74, 95% CI 1.3-5.7, p = 0.006). For both possums and bandicoots, no significant associations were found for trypanosome detections when examined against TPP, Glob, Alb, TWCC, age class or sex (p > 0.05). No seasonal association was found in haemoparasite presence in this study.

There was no detectable association with abnormal health parameters associated with Babesia, Hepatozoon, Cryptosporidium and Giardia.

3.5 Discussion

To the authors’ knowledge, this is the first identification of protozoan parasites from the genera Trypanosoma, Babesia, Hepatozoon, Cryptosporidium and Giardia in native mammals from northern Australia, using molecular methods.

Despite not being visualized in any of the blood films, trypanosomes were the most prevalent haemoproteozoa among the animals tested according to the molecular assays performed, particularly amongst bandicoots and possums. Trypanosoma vegrandis has been reported previously in a range of marsupials from WA, NSW and Queensland (Qld) (Paparini et al., 2011; Botero et al., 2013). This study reports, for the first time, the occurrence of T. vegrandis in the NT and its host range extension to include the northern brown bandicoot, providing further evidence that T. vegrandis is ubiquitous in the Australian continent.

Phylogenetic reconstructions also revealed a novel Trypanosoma genotype (NTB84) infecting bandicoots, which exhibited a relatively large genetic divergence from T. vegrandis genotype G6 at the 18S rRNA locus. This fact, combined with the poor association of NTB84 with the T. vegrandis clade, suggests that it could potentially be a novel species clustering in a monophyletic assemblage with T. copemani, T. gilletti and T. vegrandis. Additional molecular characterization is necessary to
confirm this hypothesis as reliance on 18S rDNA data alone is inadequate for species delimitation in trypanosomes (Hamilton & Stevens, 2011).

Despite recent research and greatly improved knowledge of its taxonomy and distribution, there remains limited data on the impact of *T. vegrantis* on the health of Australian marsupials. A previous study reported pathological changes in woylies co-infected with *T. vegrantis, T. noyesi* H25 and *T. copemani* (Botero et al., 2013). However, a significantly higher prevalence of *T. vegrantis* (either in single or mixed infections) in healthy woylie populations has led to the hypothesis that *T. vegrantis* could potentially moderate the pathogenicity of *T. copemani* in this host species (Botero et al., 2013; Thompson et al., 2014b).

Although no association between physical signs of disease and infection with trypanosomes was observed, analyses of blood parameters revealed that bandicoots positive for *Trypanosoma* spp. were more likely to have a lower PCV value. This finding is consistent with the results reported by McInnes et al. (2011a), who associated the presence of *T. gilletti* with lower PCV values in koalas with signs of concomitant diseases. Lower PCV values are indicative of anaemia, an abnormality associated with trypanosomiasis in animals and humans (Chisi et al., 2004; Mackie et al., 2017). Another interesting finding is that individuals with lower tooth wear scores (and likely younger) were more likely to be positive for *Trypanosoma* spp. This could be explained by the potential lower level of immunocompetence in younger animals compared to adults.

The phylogenetic position of the two novel possum-derived *T. noyesi* genotypes is consistent with previous studies showing intraspecific diversity within *T. noyesi*, and its genetic proximity to *T. cruzi*, the causative agent of human Chagas’ disease (Noyes et al., 1999; Stevens et al., 1999; Hamilton et al., 2007; Paparini et al., 2011; Botero et al., 2013; Botero et al., 2016b). The finding of
*T. noyesi* in possums from the NT provides evidence of a wider geographical distribution, as this trypanosome species had only been reported in marsupials from Vic and WA to date (Noyes et al., 1999; Paparini et al., 2011; Botero et al., 2013).

Phylogenetic analysis revealed that the *Babesia* sequences from the present study formed a separate group closely associated with *B. macropus* from eastern grey kangaroos (Dawood et al., 2013); yet the genetic distance of 1% (10 nucleotide differences) is smaller than the distance amongst other named species in the present analysis, and therefore not sufficient to define the new group as a distinct species. The paucity of morphological observation also hinders the identification of the isolates reported, as they could potentially be *B. thylacis*, for which no genetic data is available (Mackerras, 1959; Bangs & Purnomo, 1996). Moreover, no clinical signs of disease were associated with infection in bandicoots, whereas *B. macropus* appears to be highly pathogenic to its marsupial hosts (Dawood et al., 2013; Donahoe et al., 2015). Morphology associated with phylogenetic analyses based on a more variable genetic marker would help to better determine the evolutionary relationships amongst the marsupial-derived *Babesia* spp.

The molecular prevalence of *Hepatozoon* in northern brown bandicoots from the NT was lower than previous prevalence estimates reported in southern brown bandicoots from WA (51%) (Wicks et al., 2006). Gametocytes of *Hepatozoon* were only observed by microscopy in two out of 11 samples that were positive by PCR. This observation is consistent with other reports that light microscopy is less sensitive than PCR-based methods for protozoan parasite detection, especially where infection levels are low (Wicks et al., 2006; Pizarro et al., 2007; Tavares et al., 2011). Hence, we recommend the use of molecular analysis as the most effective screening method of blood samples for *Hepatozoon* sp.
This is the second published study that has employed molecular methods for the detection of *Hepatozoon* in Australian marsupials. Phylogenetic analysis revealed that all bandicoot-derived isolates grouped together, exhibiting low genetic distances from each other. Although isolates NT30 and NT31 were placed in relatively more distant positions compared to NT59 and NT72, the high genetic similarity between them and their most closely related bandicoot-derived isolate (99.6% and 99.7% respectively) suggests that all genotypes belonging to the “Australian marsupials” clade, represent small variations of the same un-named species.

The morphological features and measurements of *Hepatozoon* observed in the present study were consistent with previous descriptions of *H. peramelis*, for which no DNA sequences are available (Welsh & Dalyell, 1909; Mackerras, 1959) and subsequent isolates from other Australian marsupials (Bettiol et al., 1996; Wicks et al., 2006). Based on this and the fact that the new reported organisms did not genetically differ from the parasites described by Wicks et al. (2006), we believe that they are most likely to be *H. peramelis*. If all *Hepatozoon* sp. found in bandicoots to date are in fact *H. peramelis*, then our study provides supporting evidence that this parasite species has a wide distribution, occurring in NSW, Qld, Tasmania, WA and NT.

The prevalence of the *Cryptosporidium* sp. BTP1 in possums from the NT (0.6%) was lower than the prevalence of genotypes BTP1 and BTP2 in possums from eastern Australia (5.6-11.3%) (Hill et al., 2008). In addition, previously reported prevalence estimates of a range of *Cryptosporidium* spp. and genotypes in Australian marsupials (1.9%-19.3%) were higher compared to the present results (Power et al., 2003; Power et al., 2004; Cox et al., 2005; Power et al., 2005; McCarthy et al., 2008; Ng et al., 2011; Yang et al., 2011; Ryan & Power, 2012; Dowle et al., 2013; Nolan et al., 2013; Koehler et al., 2014; Vermeulen et al., 2015a; Koehler et al., 2016; Zahedi et al., 2016a; Zahedi et al., 2016b). The absence of clinical signs of disease associated with *Cryptosporidium* sp. BTP1
indicates a stable host-parasite relationship typical of host-adapted Cryptosporidium species (Hill et al., 2008; Power, 2010). Our study also corroborates the hypothesis that the BTP1 genotype may be host-specific and provides evidence of its wider geographic distribution. Although Cryptosporidium isolates have been found in exotic rodents (R. rattus) in the NT, raising concerns as to whether they could pose a threat to native wildlife (Paparini et al., 2012a), no rodent genotypes were detected in the animals examined, including native rodents (brush-tailed rabbit-rats).

The overall low prevalence of Giardia sp. found in native mammals from the NT is in line with the previous observation that Giardia is a relatively rare parasite in native wildlife in WA (Thompson et al., 2010). The confirmation of G. peramelis in the present study was based on sequence analysis at the 18S rRNA gene, as gdh nucleotide sequences for G. peramelis were not available (Hillman et al., 2016). The present study provides the first genetic sequences for G. peramelis at the gdh locus and phylogenetic analysis further clarifies its taxonomic position, supporting its species status and extending its host range to include common brushtail possums, northern brown bandicoots and brush-tailed rabbit-rats, contradicting previous assumptions that G. peramelis was host-specific (Adams et al., 2004; Thompson et al., 2010; Hillman et al., 2016).

In the present study, animals positive for protozoan parasites were detected at all sampling sites but in general there was no significant difference in prevalence between degraded and less degraded environments. For example, the prevalence of trypanosomes was significantly higher in Darwin compared to Kakadu, Bathurst and Groote (p = 0.05); however not significantly different from Cobourg, which is considered to have a more intact small mammal assemblage than other areas. Factors such as vector distribution and host preferences presumably play a role in the geographic distribution of these protozoans in native mammals from the NT, and therefore needs to be
investigated. Temporal and spatial analyses involving a larger sample size are also required to determine whether there is a link between specific pathogens and population declines.

Overall, the health of individuals examined did not appear to be adversely affected by infection with protozoan parasites. However, these findings do not exclude potential risk from infection, as even host-adapted parasites can pose a threat to wildlife in the presence of ecological changes and other stress factors. In addition, a lack of associated clinical signs alone is not sufficient to exclude parasitic diseases from the factors that may be contributing to the decline events in the NT. For instance, trypanosomes have been implicated in reduced fitness and increased susceptibility to predators in woylies (Thompson et al., 2014b). In this respect, the removal of parasitized and therefore compromised individuals from the population (e.g. by predation or other secondary mechanisms) could bias the results of prevalence surveys towards individuals with greater immunocompetence (and hence lower parasitism).

In conclusion, this study provides valuable baseline data on the prevalence and genetic diversity of blood-borne and enteric protozoan parasites infecting native mammal species in the NT. The knowledge generated provides a benchmark for future investigation of the spatial and temporal dynamics of parasitic infection in the NT; work which is essential to determine whether these organisms are contributing to the decline of native mammal populations. Furthermore, ongoing studies are recommended as exotic pathogenic parasites could enter Australia from the northern part of the continent and have devastating effects on highly susceptible and naïve populations.
Chapter 4  First report of *Trypanosoma vegrandis* in koalas
(*Phascolarctos cinereus*)

4.1 Preface

This chapter is a modified version of the article:

4.2 Introduction

The koala is an iconic Australian marsupial that is under threat of extinction across two thirds of its range, with population declines of over 50% being reported in some states (McAlpine et al., 2015). It is currently classified as vulnerable to extinction in New South Wales (NSW) under the NSW Threatened Species Conservation Act 1995 No 101 as Schedule 2 ‘Vulnerable species’, and in parts of Queensland under the Qld Nature Conservation (Wildlife) Regulation 2006 as Schedule 3 ‘Vulnerable wildlife’. Chlamydirosis caused by *Chlamydia pecorum* is the main disease contributing to koala population decline (Kollipara et al., 2012), acting in synergy with many other variables adversely affecting koala survival (such as concurrent infections with koala retrovirus-KoRV, habitat loss, vehicle collisions, climate change and dog attacks) (Rhodes et al., 2011; Rhodes et al., 2015).

Preliminary data suggests that trypanosome infections may also be compromising the health of wild koalas, particularly those with clinical signs of concurrent diseases, thereby also contributing to population decline events (McInnes et al., 2011a). Therefore, research on the identification, prevalence and clinical impacts of *Trypanosoma* spp. upon wild koala populations is important. In the present study we report, for the first time, the identification of *T. vegrandis* in the koala using morphological and molecular analysis.

4.3 Materials and methods

4.3.1 Sampling

A total of 44 blood samples were collected from koalas that presented to the AZWH between December 2010 and December 2011, according to standard procedures as described in chapter 2 (section 2.1).
4.3.2 Microscopy and morphometric analysis

Thin blood smears were prepared from a subset of samples and systematically examined by light microscopy as described in chapter 2 (sections 2.3.1 and 2.3.2). Digital light micrographs of any trypomastigotes observed in blood films were taken at 1,000 x magnification and then used for morphometric analysis according to the methods described in section 2.3.3.

4.3.3 Molecular analyses

Whole genomic DNA was extracted from blood samples as described in chapter 2 (section 2.4.1). A range of molecular techniques using *T. vegrandis*-specific primers and generic *Trypanosoma* primers were used to detect both *T. vegrandis* and co-infecting trypanosome species. An approximately 350 bp fragment of *T. vegrandis* 18S rDNA was amplified using a *T. vegrandis*-specific nested PCR with the forward primer TVEF and reverse primer TVER and the internal primers, TVIF and TVIR as previously described (Botero et al., 2013). Preliminary results however revealed that these primers also amplified *T. gilletti*, presumably due to the close genetic similarity between these two species. For this reason, an alternative PCR methodology was developed to amplify *T. vegrandis* from a sample (Timbo) that was positive by microscopy for *T. vegrandis*, but which was co-infected with *T. gilletti*, as initially only *T. gilletti* could be amplified using the *T. vegrandis*-specific nested PCR. Therefore, an approximately 900 bp fragment of the 18S rRNA gene was amplified using primers S823F and S662 sourced from Maslov et al. (1996), as external primers according to McInnes et al. (2009). The PCR product from these external primers was run on a 2% agarose gel stained with SYBR safe (Invitrogen, USA). The gel band was excised and purified using an in-house filter tip method as previously described (Yang et al., 2013) and 1 µl of gel-purified PCR was used in a secondary reaction with internal primers TVIF and TVIR (Botero et al., 2013). In order to detect co-infections with other trypanosome species, all samples that were positive for *T. vegrandis* using the *T. vegrandis*-specific primers were also screened by nested PCR.
using generic 18S rDNA *Trypanosoma* primers (TRY927F, TRY927R, SSU561F and SSU561R) as previously described (Noyes et al., 1999).

All positive PCR products obtained using *T. vegrandis*-specific PCR and universal trypanosome PCR were purified and sequenced using the corresponding internal primers, as described in chapter 2 (sections 2.4.3 and 2.4.4). Nucleotide sequences obtained in this investigation were deposited in GenBank under the accession numbers KP271047- KP271052.

**4.3.4 Phylogenetic analysis**

Phylogenetic analysis and estimation of genetic distances between sequences were conducted as described in chapter 2 (section 2.5). In the present study, the evolutionary history was inferred using the Tamura-Nei substitution model (Tamura & Nei, 1993). The model was selected assuming an estimated proportion of invariant sites and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data.

**4.4 Results**

**4.4.1 Microscopy and morphometric analysis**

Using light microscopy, the morphology of *T. vegrandis* in blood films from the koala comprised short stumpy lanceolate-shaped trypanosomes, with no undulating membranes (Fig. 4.1). The trypanosomes generally lacked a clearly defined kinetoplast and nucleated region and were observed as polymorphic. The posterior end was long and drawn out and tapered into a point. The anterior end was more rounded and contained a short free flagellum. The measurable morphological dimensions of the two trypanosomes in the koala (Fig. 4.1a) ranged in total length between 5.8 - 8.29 µm and in width between 0.9 - 1.3 µm. The measurable morphological dimensions of the larger trypanosome, which contained a faintly stained nuclear region (Fig. 4.1b) was 9.98 µm in
total length, 1.79 µm in width, 6.91 µm from posterior to nucleus, 2.85 µm from nucleus to anterior and 4.17 µm in free flagellum.

Figure 4.1. Light photomicrograph of the trypomastigote stages of *T. vegrandis* in a Modified Wright’s stained blood film from a koala (isolate Timbo). Scale bars represent 10µm.

4.4.2 Prevalence of *Trypanosoma vegrandis* in koalas

The PCRs conducted on the 18S rRNA locus using *T. vegrandis*-specific internal primers confirmed the presence of *T. vegrandis* in 6 out of 44 koalas examined, a total prevalence of 13.6% (95% CI: 5.2-27.4%).

The universal trypanosome nested PCR by Noyes et al. (1999) revealed that 3 isolates (K10, K55, K59) were also positive for *T. irwini* and mixed chromatograms were obtained for samples K9 and K37. The presence of *T. gilletti* was confirmed in isolate Timbo, which had originally been detected as positive for this trypanosome species using the *T. vegrandis* species-specific nested PCR as reported in section 4.3.3.
4.4.3 Phylogenetic analysis

Phylogenetic analysis of the relationships between the *T. vegrandis* isolates detected in the present study with *T. vegrandis* 18S rDNA genotypes on GenBank and a range of other trypanosome species, confirmed that all the sequences isolated from koalas belonged to the *T. vegrandis* clade (Fig. 4.2). Concordant phylogenies were obtained using both neighbour-joining (data not shown) and maximum likelihood approaches. There was extensive genetic diversity amongst *T. vegrandis* isolates from koalas and other hosts which exhibited between 0% and 6.3% genetic distance from each other with the largest genetic distance (6.3%) between two koala-derived isolates (K9 and Timbo). Two isolates (Timbo and K55) exhibited 100% similarity to *T. vegrandis* isolate G7 (KC753536) and two (K9 and K10) were 100% identical to *T. vegrandis* isolate G6 (KC753535), both of which were from woylies. Two isolates (K37 and K59) grouped within the *T. vegrandis* clade but exhibited 3 and 4 single nucleotide polymorphisms (SNP’s) from *T. vegrandis* isolate G7 (KC753536), respectively.
Figure 4.2. Maximum likelihood analysis of the relationships between Trypanosoma spp. and koala-derived T. vegrandis at the 18S rRNA locus.
4.5 Discussion

This is the first report of *T. vegrandis* in koalas, a finding that was confirmed using morphology and molecular analysis. In the present study, a clearly defined kinetoplast could not be observed in the trypomastigotes probably due to the small size of *T. vegrandis*, which can limit an accurate identification of internal structures, when compared to larger trypanosome species. However, the images and morphological dimensions presented in this study are clearly similar to the *T. vegrandis* trypomastigotes described by Thompson et al. (2013). Although the isolate (Timbo) examined by microscopy had a mixed *T. vegrandis/T. gilletti* infection, the trypanosomes measured are much too small to be *T. gilletti*, which has been speculated to measure 47.1 µm in length and 1.2 µm in breadth (McInnes et al., 2011b). The identity of *T. vegrandis* was also confirmed using sequence analysis of the 18S rRNA gene, which clearly showed that the koala-derived isolates grouped within the *T. vegrandis* clade.

Mixed trypanosome infections are common in koalas (McInnes et al., 2011a) and it has been reported that the use of generic PCR to detect trypanosome infections can mask the presence of less abundant genotypes (McInnes et al., 2011a; Botero et al., 2013). It is likely that the small size of *T. vegrandis* (< 10 µm in length), coupled with the difficulties in amplifying this parasite in mixed infections using generic trypanosome primers, are the reason why the parasite has not been identified until now in koalas.

This is the first time that *T. vegrandis* species-specific primers have been used on blood from koalas. Initial attempts to amplify *T. vegrandis* using primers that were reported to be specific to *T. vegrandis* (Botero et al., 2013), amplified *T. gilletti*, which is phylogenetically most closely related
to *T. vegrandis* (Paparini et al., 2011; Botero et al., 2013). As a result of this, an alternative methodology was developed to amplify *T. vegrandis* using a generic trypanosome external primer set (Maslov et al., 1996), cutting out the resultant PCR band from the gel and then using 1 µl of the purified gel slice with the *T. vegrandis* specific primers, for a second round of PCR. The method worked well and may be useful for amplifying *T. vegrandis* from other mixed infections. In the present study, screening of the *T. vegrandis* positive isolates with the universal trypanosome nested PCR by Noyes et al. (1999), provided evidence of mixed infections involving *T. vegrandis* and one or more trypanosome species (*T. gilletti* or *T. irwini*). This finding highlights the importance of the use of *T. vegrandis* specific primers and sequencing the products for identification of this parasite species in naturally infected koalas.

The PCR prevalence of *T. vegrandis* in koalas (13.6%; 95% CI: 5.2-27.4%) reported in the present study was at the lower end of prevalence estimates of *T. vegrandis* previously reported in woylies (14% - 46%) (Smith et al., 2008; Botero et al., 2013; Thompson et al., 2014b) and various other marsupial species (up to 32%) (Botero et al., 2013) and bat species (88.9%) (Austen et al., 2015a). In koalas, *T. vegrandis* was significantly less prevalent than *T. irwini* (71.1%), but more prevalent than *T. copemani* (4.4%) (McInnes et al., 2011b). The group of animals tested in the present study comprised presumably sick or injured koalas presenting to the AZWH and therefore may not reflect the prevalence in the wild population. Further investigations comprising a larger sample size and random sampling are required to determine and compare the prevalence more accurately. Differences in prevalence could also be due to different locations, hosts, sensitivity of molecular tests used and the capacity of Australian trypanosomes to migrate to different organs in the host (Botero et al., 2013).
The observation that *T. vegrandis* is present in marsupials and bats from WA and NSW (Averis et al., 2009; Paparini et al., 2011; Botero et al., 2013; Thompson et al., 2013; Austen et al., 2015a), marsupials in the NT (chapter 3) and now in koalas from Qld suggests that this parasite species has a wide distribution in Australia. Of the eight native trypanosomes formerly described, only *T. copemani* and *T. vegrandis* species have been identified in multiple hosts. The current findings add more evidence to the fact that trypanosomes in Australian marsupials comprise a heterogeneous community, with low levels of host specificity and no evidence of restricted geographical distribution (Noyes et al., 1999; Smith et al., 2008; Austen et al., 2009; Paparini et al., 2011; Botero et al., 2013; Thompson et al., 2014a; Thompson et al., 2014b).

Little is known about the clinical significance of *Trypanosoma* spp. in Australian native mammals. However, it is noteworthy that *T. gilletti* has been implicated in the decreased survival of koalas with signs of concomitant diseases; and *T. copemani* has been associated with anaemia in this marsupial (McInnes et al., 2011a). Additionally, *T. copemani* has been associated with pathological changes such as muscle degeneration in woylies and may be implicated in the decline of woylie populations in WA (Botero et al., 2013; Thompson et al., 2014b). Further research is required to determine the prevalence and potential impacts of *T. vegrandis* and mixed trypanosome infections on koala health.
Chapter 5  

Increased genetic diversity and prevalence of co-infection with *Trypanosoma* spp. in koalas (*Phascolarctos cinereus*) and their ticks identified using Next-generation Sequencing (NGS)

5.1 Preface

This chapter is a modified version of the article:

5.2 Introduction

A range of factors, including infectious diseases, have been associated with significant koala population declines that have been reported in some Australian states (see section 4.3). Four *Trypanosoma* spp. in either single or mixed infections have been recorded in koalas to date: *T. irwini*, *T. gilletti*, *T. copemani* (McInnes et al., 2009; McInnes et al., 2011a) and *T. vegrandis* (chapter 4), at prevalences ranging from 4.4% to 71.1%. Previous research has shown that trypanosomes were associated with indicators of koala ill-health and non-survival (McInnes et al., 2011a).

Although NGS is a well-established method for profiling bacterial communities, with the exception of *Plasmodium* in mosquitoes, relatively few studies have applied this technology in the diagnosis of protozoal infections (Paparini et al., 2015a; Vermeulen et al., 2016; Paparini et al., 2017; Zahedi et al., 2017). To the best of the authors’ knowledge, no previous published studies have used an NGS approach to investigate mixed trypanosome infections in wildlife, particularly koalas, or in potential vectors such as ticks. The development of such protocol is therefore critical, not only from a conservation awareness perspective, but also in a One Health context, as NGS has also the potential to uncover zoonotic pathogens that may be present in relatively low abundance within wildlife and tick samples.

The present study aimed to design and validate a novel NGS-based assay targeting the 18S rRNA locus to audit *Trypanosoma* communities in koala blood samples and ticks removed from koala hosts. This NGS assay was then used to determine the prevalence and genetic diversity of *Trypanosoma* sp. in koalas from eastern Australia and identify potential vectors for *Trypanosoma*. 
5.3 Materials and methods

5.3.1 Sampling

A total of 168 blood samples and 91 ticks collected from koalas were investigated during the present study. Blood samples were collected during routine clinical procedures (as described in section 2.1) from 161 koalas (84 females and 77 males) admitted to the AZWH between December 2010 and December 2011. An additional seven blood samples were collected from koalas (three females and four males) that presented to the Koala Hospital, Port Macquarie, NSW, between October 2014 and February 2015.

Of the 91 ticks collected from koalas between December 2009 to August 2014; 81 were collected at the AWZH and the remaining 10 were supplied by Endeavour Veterinary Ecology Pty Ltd, Toorbul, Qld. The ticks were stored in 70% ethanol after collection as described in section 2.1. Due to differences in sampling sites, collection dates, and protocols (i.e. ticks were collected opportunistically and blood was collected during routine veterinary procedures that required anaesthesia), the ticks and blood from corresponding hosts (i.e. koala parasitized by that particular tick) were collected concurrently in only 15 cases.

5.3.2 Tick identification

Tick instars and species were morphologically identified using the methodology described in section 2.2. All ticks analysed in the present study belonged to the family Ixodidae; of which 56 (61.5%) were identified as *I. tasmani* and 35 (38.5%) as *I. holocyclus*. In total, 82 (93.8%) of ticks were females. Of these, 49 (59.8%) belonged to the species *I. tasmani* and 33 (40.2%) to the species *I. holocyclus*. Only 9 ticks (7 *I. tasmani* and 2 *I. holocyclus*), were males. No nymphs or larvae were observed.
5.3.3 DNA Extraction

Genomic DNA was isolated from whole blood and ticks according to the procedures reported in chapter 2 (section 2.4.1).

5.3.4 Trypanosoma 18S rRNA gene metabarcoding

Partial Trypanosoma 18S rRNA gene sequences (~350 bp) were PCR amplified from koala and tick DNA samples using a hemi-nested PCR assay utilizing the primary primers S825F (Maslov et al., 1996) and TryAll R1 (5’-GACTGTAACCTCAAGCCTTTCGC-3’) (designed during the present study), and hemi-nested primers S825F and S662R (Maslov et al., 1996). Hemi-nested PCR primers also contained Illumina MiSeq adapter sequences on the 5’ end, as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocols: Metagenomic Sequencing Library Preparation). PCRs were performed in 25 µl volumes containing PCR buffer, 1.5 mM MgCl₂, 1 mM dNTPs, 0.8 µM of each primer, 0.04 U/ml Taq DNA polymerase (Fisher Biotec, Australia) and 2 µl of DNA (primary PCR).

Primary PCR products were electrophoresed through a 2% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA), visualized with a dark reader trans-illuminator (Clare Chemical Research, USA), and products corresponding to the correct length were excised, purified using a QIAquick gel extraction kit (QIAGEN, Germany). Hemi-nested PCRs used 1 µl of purified primary product as a template. Primary and hemi-nested PCRs were performed with an initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C (primary PCR) or 55 °C (hemi-nested PCR) for 30 s, and extension at 72 °C for 30 s, followed by a final extension of 72 °C for 5 min. Hemi-nested PCR products were electrophoresed and purified as for the primary PCR products.
DNA samples from seven koalas (previously obtained from the AZWH) and known to harbour dual or triple trypanosome infections with *T. irwini*, *T. gilletti* and *T. copemani* (as determined by Sanger sequencing of cloned PCR products and/or species-specific PCR’s) (McInnes et al., 2011a; McInnes et al., 2011b), were included in this experiment as positive controls. In addition, DNA from the pathogenic *T. cruzi* was included as a representative of a distantly related species from those known to infect koalas, to make sure primers were able to pick up a wide range of species including those within this important clade of trypanosomes. No-template and extraction blank controls were included in all PCR assays.

Resulting *Trypanosoma* 18S rDNA amplicons from each sample were then uniquely indexed with DNA barcodes and prepared for sequencing according to Illumina recommended protocols (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation), and sequenced on an Illumina MiSeq using 500-cycle V2 chemistry (250 bp paired-end reads), following the manufacturer’s recommendations.

**5.3.5 Bioinformatics analysis**

Paired-end reads were overlapped (50 bp minimum overlap length, no mismatches allowed) and merged in USEARCH v8.0.1623 (Edgar, 2010). Sequences were then imported into Geneious 8.0.4 (Kearse et al., 2012), where S825F and S662R primer sequences and distal bases were trimmed from the 5’ and 3’ ends of the reads. Only sequences containing the correct primer sequences (no SNPs) were retained for further analyses. In USEARCH v8.0.1623, the sequences were quality filtered (sequences with >2% expected errors were excluded from the dataset), de-multiplexed and singletons were removed on a per-run basis.
Trypanosoma operational taxonomic units (OTUs) were generated by clustering sequences with $\geq 99\%$ similarity, using the UPARSE algorithm (Edgar, 2013). This threshold was determined based on the smallest genetic divergence at the 18S rRNA locus studied between trypanosome species found in koalas to date, according to an in-house database comprising curated Trypanosoma 18S rDNA sequences retrieved from GenBank. A list of species, isolate names and respective GenBank accession codes are shown in Table 5.1.

**Table 5.1. GenBank accession numbers of Trypanosoma 18S rDNA sequences used as reference for NGS taxonomic assignment and phylogenetic analysis.**

<table>
<thead>
<tr>
<th>Trypanosome species or isolate name</th>
<th>GenBank accession code</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. cruzi</em> Y</td>
<td>AF301912</td>
</tr>
<tr>
<td><em>T. cruzi</em> Tcbat</td>
<td>FJ900241</td>
</tr>
<tr>
<td><em>T. cruzi</em> marinkellei</td>
<td>FJ001664</td>
</tr>
<tr>
<td><em>T. cruzi</em> G marsupial</td>
<td>AF239981</td>
</tr>
<tr>
<td><em>T. rangeli</em></td>
<td>FJ900242</td>
</tr>
<tr>
<td><em>T. minasense</em></td>
<td>AJ012413</td>
</tr>
<tr>
<td><em>T. dionisii</em></td>
<td>FJ001666</td>
</tr>
<tr>
<td><em>T. erneyi</em></td>
<td>JN040987</td>
</tr>
<tr>
<td><em>T. vespertilionis</em></td>
<td>AJ009166</td>
</tr>
<tr>
<td><em>Trypanosoma</em> 1 EA-2008</td>
<td>FM202492T</td>
</tr>
<tr>
<td><em>T. livingstonei</em></td>
<td>KF192979</td>
</tr>
<tr>
<td>Trypanosome species or isolate name</td>
<td>GenBank accession code</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td><em>T. conorhini</em></td>
<td>AJ012411</td>
</tr>
<tr>
<td><em>T. sp. NanDoum1</em></td>
<td>FM202492</td>
</tr>
<tr>
<td><em>T. sp. HochNdi1</em></td>
<td>FM202493</td>
</tr>
<tr>
<td><em>T. noy esi H25</em></td>
<td>AJ009168</td>
</tr>
<tr>
<td><em>T. noyesi AP-2011-64</em></td>
<td>JN315383</td>
</tr>
<tr>
<td><em>T. noyesi G8</em></td>
<td>KC753537</td>
</tr>
</tbody>
</table>

After chimera filtering was carried out, the retained OTUs were BLAST-searched against the same in-house trypanosome library. Taxonomy was assigned to a species level in QIIME v.1.9.1 (Caporaso et al., 2010), using the BLAST algorithm (e = 0.0001). The sequences not assigned to a species level were classified as either “*Trypanosoma* sp.” (i.e. assigned to a genus-level only) or “no BLAST hits” (i.e. unassigned), and further inspected during the phylogenetic analysis.

In order to design an effective taxonomic assignment method for the present study, several algorithms and stringency parameters available in QIIME v.1.9.1 were tested, starting from highly stringent protocols (e.g. BLAST with 100% similarity threshold; BLAST with 99% similarity threshold associated with a 2/3 consensus fraction using the UCLUST algorithm). To verify the sensitivity and specificity of the algorithm selected upstream, fast phylogenetic trees were drawn in Geneious 8.0.4 (function: Fast Tree), using all assigned and unassigned OTU sequences, after each batch of test results was generated (data not shown). A number of incongruences between the NGS-BLAST taxonomic assignment and taxonomy revealed by phylogenetic analysis were observed.
when using more stringent parameters. For instance, many sequences classified as “unassigned” due to a lower genetic identity compared to a determined reference species (< 98%), could be assigned to species according to the phylogenetic reconstructions. Therefore, the method was refined and a relatively less conservative parameter that would simply choose the BLAST “top hit” (e = 0.0001) was selected as the most reliable approach to reflect the trypanosome diversity and sequence abundance within the samples.

To exclude sampling depth heterogeneity, alpha-rarefaction plots were generated in QIIME (rarefaction was set at 14,136 and 11,158 sequence reads for koalas and ticks, respectively). The graphs suggested that a satisfactory sampling depth had been obtained to accurately represent the trypanosome community present within the samples, as curves appeared to plateau after about 2,000 reads (data not shown).

NGS data obtained during the present study is available under NCBI BioProject ID: 2588872 (Accession No. PRJNA383324).

5.3.6 Phylogenetic analysis

Phylogenetic analysis was conducted on a selection of OTUs and 18S rDNA *Trypanosoma* reference sequences retrieved from GenBank. OTUs assigned to a species level that were representative of a relatively greater number of reads were selected; of which, one representative of each *Trypanosoma* sp. identified in the current study, from both koalas and ticks, were included in the phylogenetic analysis. All “unassigned” and “assigned to a genus-level only” OTUs were also added to the dataset for inspection.

Evolutionary analyses were conducted on 51 sequences using the resources of the software MEGA 6, as described in chapter 2 (section 2.5). In the present study, the evolutionary history was inferred
based on the Kimura 2-parameter model (Kimura, 1980), using uniform rates and 95% partial deletion. All “unassigned” OTUs confirmed by phylogenetic analysis to be the result of non-specific amplifications were removed from the dataset, the analysis repeated and a final tree generated. Estimates of genetic distances between sequences were conducted using the Kimura 2-parameter model, in MEGA 6.

*Trypanosoma* 18S rDNA sequences of selected OTUs included in the phylogenetic analysis were submitted to GenBank under the following accession numbers: KX786142-KX786149 and KY640309-KY640322.

### 5.3.7 Sanger sequencing of *Trypanosoma* 18S rDNA

For Sanger sequencing, the internal forward and reverse primers S825F and S662R (Maslov et al., 1996) used in the hemi-nested PCR, but without the MiSeq adapters, were used in a single-round PCR assay. The resultant amplicons were separated on a 2% agarose gel, purified and then Sanger sequenced in both directions according to the methods described in chapter 2 (sections 2.4.3 and 2.4.4). The sequences were BLAST-searched online against the GenBank nucleotide database (https://blast.ncbi.nlm.nih.gov). The results provided by Sanger and NGS methods were compared.

### 5.4 Results

#### 5.4.1 Sanger sequencing analysis

In total, 59 out of 168 koala blood samples (35.1%, 95% CI: 27.9-42.8%) were positive for *Trypanosoma* DNA by single-round PCR. Sanger sequencing of amplicons obtained from these 59 samples revealed the presence of three species: *T. irwini*, *T. gilletti* and *T. copemani*, at prevalences of 28.6% (95% CI: 21.9-36%), 1.2% (95% CI: 0.1-4.2%) and 5.4% (95% CI: 2.5-9.9%), respectively.
A total of 23 ticks were positive by single-round PCR, however specific *Trypanosoma* DNA sequences were only obtained for 21 of these samples by Sanger sequencing (23.1%, 95% CI: 14.9-33.1%). Only two species (*T. gilletti* and *T. copemani*) were identified within ticks using Sanger sequencing, at prevalences of 17.6% (95% CI: 10.4-27%) and 5.5% (95% CI: 1.8-12.4%), respectively. No amplicons were detected after the PCR of non-template control and extraction blank control.

### 5.4.2 NGS bioinformatics analysis

A total of 3,565,056 paired-end reads were obtained from 61 out of 175 (34.9%) koala blood samples (including the positive controls) and 23 out of 91 (25.3%) ticks. Of the total of number of sequences, 3,475,280 (97.5%) contained the correct primer sequences (no SNPs) and were retained for further analyses. During the FASTQ filtering process, 97% of the reads were retained and 3% were excluded from the dataset due to their low quality. A total of 5,990 OTUs were created during the OTU clustering step, of which 92.8% passed chimera filtering. Taxonomy was assigned to 5,562 OTUs, whilst 15 OTUs had no BLAST hits.

### 5.4.3 Overall prevalence and molecular characterisation of *Trypanosoma* spp. by NGS

In total, 54 out of 168 koalas (32.2%, 95% CI: 25.2-39.8%) and 23 out of 91 ticks (25.3%, 95% CI: 16.7-35.5%) were positive for *Trypanosoma* spp. by NGS. The prevalence estimates of trypanosomes in *I. holocyclus* (34.3%, 95% CI: 19.1-52.2%) and *I. tasmani* (19.7%, 95% CI: 10.2-32.4%) were not significantly different (p > 0.05). *Trypanosoma* infection profiling by NGS analysis revealed the presence of five *Trypanosoma* spp. (*T. irwini*, *T. gilletti*, *T. copemani*, *T. vegrandis* and *T. noyesi*) within koala blood samples. The same trypanosome species were detected in ticks, except for *T. noyesi*. Additionally, a novel species classified only as *Trypanosoma* sp. by NGS analysis was present in DNA samples from koalas, *I. holocyclus* and *I. tasmani*. All positive
controls produced identical results to those obtained in previous studies, which used cloning of PCR products and species-specific PCRs to identify mixed trypanosome infections (McInnes et al., 2011a; McInnes et al., 2011b). No trypanosome sequences were obtained from the non-template and extraction blank controls.

Phylogenetic analysis performed on a subset of NGS OTUs, representative of a relatively greater number of sequences isolated from koalas and ticks, produced concordant phylogenetic tree topologies using the neighbour-joining (data not shown) and maximum likelihood methods (Fig. 5.1). The analysis confirmed the OTU’s molecular identity during the taxonomic assignment process. Produced concordant phylogenetic trees were obtained using both neighbour-joining (data not shown) and maximum likelihood methods (Fig. 5.1). Phylogenetic reconstructions also revealed that the NGS method was able to discriminate distinct genotypes of *T. copemani* (G1 and G2) and *T. vegrandis* (G5-G7 and AP-2011b-28 clone 11). The pairwise identity match between OTUs and their corresponding reference sequences (i.e. species they were identified as) ranged from 97% to 100%, according to the estimates of genetic divergence matrix (data not shown). Genetic variations of up to 7% within genotypes of the same species were observed.
Figure 5.1. Phylogenetic analysis of a selection of NGS Operational Taxonomic Units (OTUs) assigned as *Trypanosoma* spp. and reference trypanosome sequences retrieved from GenBank. The analysis was based on 18S rDNA partial sequences (~350 bp), using the maximum likelihood method. Bootstrap values (>60%) are indicated at the left of each node.
OTUs assigned to a genus-level only in the NGS pipeline (hereafter referred as *Trypanosoma* sp. AB-2017), clustered together and formed a separate clade strongly supported by bootstrap values (Fig 5.1). This clade formed a sister clade to the group consisting of *T. copemani*, *T. gilletti* and *T. vegrandis*. Minor genetic divergence (1-2%) was observed between the sequences within the novel clade.

5.4.4 Molecular prevalence and genetic diversity of *Trypanosoma* spp. in koalas and ticks

The prevalence of each trypanosome species detected by NGS in koalas and ticks (*I. tasmani* and *I. holocyclus*), is presented in Fig 5.2. In koalas, the prevalence of *T. irwini* (32.1%, 95% CI: 25.2-39.8%), *T. gilletti* (25%, 95% CI: 18.7-32.3%) and *T. copemani* (27.4%, 95% CI: 20.8-34.8%) was significantly higher compared to the prevalence of *T. vegrandis* (10.1%, 95% CI: 6.0-15.7%), *T.
Trypanosoma noyesi (0.6%, 95% CI: 0-3.3%) and novel Trypanosoma sp. AB-2017 (4.8%, 95% CI: 2.1-9.2%). No significant difference was observed among prevalences of the three, dominant species (p > 0.05).

Trypanosoma irwini, T. gilletti and T. copemani were also the three most frequently identified species in I. holocyclus, with prevalence estimates of 34.3% (95% CI: 19.1-52.2%), 31.4% (95% CI: 16.9-49.3%) and 34.3% (95% CI: 19.1-52.2%), respectively. However, the prevalence of each Trypanosoma spp. identified from ticks were not statistically different from each other (p > 0.05).

In I. tasmani ticks, even though the prevalence estimates for T. gilletti and T. copemani (19.6%, 95% CI: 10.2-32.4%, each) were relatively higher, they were not significantly different from those of T. irwini and T. vegrandis (12.5%, 95% CI: 5.2-24.1%, each). No major differences were observed in the pattern of Trypanosoma spp. prevalence when comparing koalas to ticks (partially matched data) or both tick species to each other (Fig. 5.2).

The prevalence of each genotype of T. copemani and T. vegrandis identified in koalas and ticks was also calculated, considering the potential differences in pathogenicity and/or virulence that might exist between these distinct variants (Table 5.2). In koalas, the prevalence of sequences classified as T. copemani isolate Charlton was significantly higher than those of T. copemani G1 and G2 (p < 0.05). As for T. vegrandis, G5 and G6 were the most common genotypes found in koalas; of which G6 showed a significantly higher prevalence compared to G3, G4, G7, AP-2011b-4c6 and AP-2011b-28c11 (p < 0.05). In contrast, all genotypes of T. copemani and T. vegrandis were present in statistically similar prevalences within I. holocyclus and I. tasmani (p > 0.05).
Table 5.2. Prevalence of known genotypes of *T. copemani* and *T. vegrandis* in koalas and ticks (*I. holocyclus* and *I. tasmani*), determined by NGS at the 18S rRNA locus.

<table>
<thead>
<tr>
<th>Trypanosome</th>
<th>Genotype (GenBank accession code)</th>
<th>Koala</th>
<th><em>I. holocyclus</em></th>
<th><em>I. tasmani</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. copemani</em></td>
<td>Charlton (GU966588)</td>
<td>27.4 (20.8-34.8)(^a)</td>
<td>8.2 (2.3-19.6)(^a)</td>
<td>9.5 (4.4-17.2)(^a)</td>
</tr>
<tr>
<td></td>
<td>G1 (KC753530)</td>
<td>1.2 (0.1-4.2)(^b)</td>
<td>10.2 (3.4-22.2)(^a,b)</td>
<td>8.4 (2.3-15.9)(^a,b)</td>
</tr>
<tr>
<td></td>
<td>G2 (KC753531)</td>
<td>4.8 (2.1-9.2)(^b)</td>
<td>18.4 (8.8-32)(^a)</td>
<td>9.5 (4.4-17.2)(^a)</td>
</tr>
<tr>
<td><em>T. vegrandis</em></td>
<td>G3 (KC753533)</td>
<td>0 (0-2.2)(^b)</td>
<td>0 (0-7.3)(^b)</td>
<td>0 (0-3.8)(^b)</td>
</tr>
<tr>
<td></td>
<td>G4 (KC753532)</td>
<td>0 (0-2.2)(^b)</td>
<td>0 (0-7.3)(^b)</td>
<td>0 (0-3.8)(^b)</td>
</tr>
<tr>
<td></td>
<td>G5 (KC753534)</td>
<td>4.2 (1.7-8.4)(^b,c)</td>
<td>0 (0-7.3)(^b)</td>
<td>0 (0-3.8)(^b)</td>
</tr>
<tr>
<td></td>
<td>G6 (KC753535)</td>
<td>7.1 (3.7-12.1)(^c)</td>
<td>0 (0-7.3)(^b)</td>
<td>0 (0-3.8)(^b)</td>
</tr>
<tr>
<td></td>
<td>G7 (KC753536)</td>
<td>0.6 (0-3.3)(^b)</td>
<td>0 (0-7.3)(^b)</td>
<td>0 (0-3.8)(^b)</td>
</tr>
<tr>
<td></td>
<td>AP-2011b-4c6 (JN315392)</td>
<td>0 (0-2.2)(^b)</td>
<td>10.2 (3.4-22.2)(^a,b)</td>
<td>7.4 (3-14.6)(^a,b)</td>
</tr>
<tr>
<td></td>
<td>AP-2011b-28c11 (JN315387)</td>
<td>0.6 (0-3.3)(^b)</td>
<td>0 (0-7.3)(^b)</td>
<td>0 (0-3.8)(^b)</td>
</tr>
</tbody>
</table>

Values in the same column followed by different letters are statistically distinct (p < 0.05)

Among all koalas positive for *Trypanosoma* DNA by NGS (n = 54, excluding the positive controls), 34 (63%) were females and 20 (37%) were males. Overall, no significant difference between the prevalence of trypanosomes in koala females (39.1%, 95% CI: 28.8-50.1%) and males (24.7%, 95% CI: 17.2-33.9%) was observed.
CI: 15.8-35.5%) was observed. *Trypanosoma* DNA was only detected by NGS in female ticks; however, this result should be cautiously interpreted as the sample size of male ticks (n = 9) was considerably smaller than of females (n = 82).

5.4.5 Characterization of single and mixed trypanosome infections

Intra-individual trypanosome mixed infections were observed in koalas, *I. holocyclus* and *I. tasmani*, at relatively higher prevalences compared to infections involving a single species (Fig. 5.3).

![Figure 5.3. Prevalence of single and mixed trypanosome infections in koalas, *I. holocyclus* and *I. tasmani*, determined by NGS. Error bars represent 95% confidence intervals (95% CI).](image)
Co-infections with up to five different species were observed in koalas and ticks, not considering the genotype diversity within *T. copemani* and *T. vegrandis*. The composition of these mixed infections, in relation to the number of different species involved, is shown in Fig. 5.4. Infections involving three and four *Trypanosoma* spp. were more frequent in koalas, whereas in ticks, the majority of samples with mixed infections, harboured four trypanosome species.

![Doughnut charts](chart.png)

**Figure 5.4.** Characterisation of mixed trypanosome infections in koalas (A), *I. holocyclus* (B) and *I. tasmani* (C), based on the number of unique trypanosome species involved. Each doughnut chart shows proportions of the total number (n) of samples harbouring more than one *Trypanosoma* sp., based on the NGS analysis.

Most mixed infections with three species in koalas consisted of co-infections with *T. irwini*, *T. gilletti*, and *T. copemani* at a prevalence of 12.5% (95% CI: 7.9%-18.5%). Of all cases of mixed infections involving four *Trypanosoma* spp., coinfections involving *T. irwini*, *T. gilletti*, *T. copemani* and *T. vegrandis* were the most prevalent (7.7%, 95% CI: 4.2-12.8%). *Trypanosoma irwini* was the parasite identified in all cases of single infection in koalas.
In *I. holocyclus*, cases of multiple infections involving *T. irwini*, *T. gilletti*, *T. copemani* and *T. vegrandis* were the most prevalent (27.3%, 95% CI: 13.3%-45.5%). The majority of *I. tasmani* ticks that were positive for *Trypanosoma* DNA also harboured mixed infections with these four trypanosome species (prevalence: 12.2%, 95% CI: 4.6%-24.8%); however, co-infections with *T. gilletti*, *T. copemani* and *T. vegrandis* were relatively common among the positives (prevalence: 8.2%, 95% CI: 2.3%-19.9%).

5.4.6 NGS coverage and *Trypanosoma* abundance

In total, 3,027,379 reads obtained from koala blood and ticks, using NGS, were identified as *Trypanosoma* sp. This total includes the OTUs assigned to a genus or species level and the sequences they clustered with during the OTU clustering step. Table 5.3 displays the total number and proportion of NGS reads assigned to each *Trypanosoma* sp. detected in koalas, *I. holocyclus* and *I. tasmani*. Overall, in koalas, a remarkably high proportion of the reads (79%) were identified as *T. irwini*. In contrast, over 99.8% of the sequences obtained from *I. holocyclus* were taxonomically assigned as *T. gilletti*. A high number of copies identified as *T. gilletti* and *T. copemani* were isolated from *I. tasmani*, which represented 50% and 46% of the total, respectively. The proportion of sequences identified as *T. irwini* in both tick species investigated (up to 3.1% only) was significantly lower compared to koalas (p < 0.05).

Table 5.3. NGS coverage and relative abundance of taxonomically assigned sequence reads obtained from koalas and ticks (*I. holocyclus* and *I. tasmani*).

<table>
<thead>
<tr>
<th>Trypanosoma spp.</th>
<th>Koala (n = 61*)</th>
<th><em>I. holocyclus</em> (n = 12)</th>
<th><em>I. tasmani</em> (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sequences</td>
<td>%</td>
<td>Number of sequences</td>
<td>%</td>
</tr>
<tr>
<td><em>T. irwini</em></td>
<td>2,052,257</td>
<td>79.702</td>
<td>87</td>
</tr>
<tr>
<td>Trypanosoma spp.</td>
<td>Koala (n = 61*)</td>
<td>I. holocyclus (n = 12)</td>
<td>I. tasmani (n = 11)</td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
<td>-----------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td></td>
<td>Number of sequences</td>
<td>%</td>
<td>Number of sequences</td>
</tr>
<tr>
<td>T. gilletti</td>
<td>254,721</td>
<td>9.892</td>
<td>234,427</td>
</tr>
<tr>
<td>T. copemani</td>
<td>251,770</td>
<td>9.778</td>
<td>164</td>
</tr>
<tr>
<td>T. vegrandis</td>
<td>9,897</td>
<td>0.384</td>
<td>72</td>
</tr>
<tr>
<td>Trypanosoma sp. AB-2017</td>
<td>6,247</td>
<td>0.243</td>
<td>26</td>
</tr>
<tr>
<td>T. noyesi</td>
<td>36</td>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>2,574,928</td>
<td>100</td>
<td>234,776</td>
</tr>
</tbody>
</table>

*54 positive samples + 7 positive controls

The NGS data analysis revealed that *T. vegrandis*, *Trypanosoma* sp. AB-2017 and *T. noyesi* (detected in koalas only) produced a considerably lower amount of sequence reads overall compared to *T. irwini*, *T. gilletti* and *T. copemani*. However, analysis of sequence profiles per sample revealed that these presumably rare species occurred in more than one sample, with corresponding number of reads ranging from 12 to 9,463, per sample. This provides supporting evidence that the reads were true sequences (i.e. not originated from possible sequencing errors).

To examine intra-individual genetic variation of the parasites, excluding the sampling depth heterogeneity, a composition plot was generated from rarefied data to represent the relative proportion of multiple genotypes circulating within each positive koala sample (Fig. 5.5) and tick
The analysis shows that 100% of the koalas positive for *Trypanosoma* DNA were infected with *T. irwini*, and also confirms the relatively higher abundance of *T. irwini* sequences within most koala samples positive by NGS. The results also show a relatively high frequency of *T. gilletti* in ticks, which was the dominant trypanosome species within the invertebrate hosts.

Figure 5.5. Composition plot illustrating the trypanosome infection profile of each positive koala sample at the 18S rRNA locus, by NGS. Rarefaction was set at 14,136 sequences.
Figure 5.6. Composition plot illustrating the trypanosome infection profile of each positive tick sample at the 18S rRNA locus, by NGS. Rarefaction was set at 11,158 sequences.

5.4.7 Comparison of NGS and Sanger sequencing-based assay performances

A comparison between prevalence estimates of *Trypanosoma* spp. in koalas and ticks, obtained using NGS and Sanger sequencing is presented in Table 5.4.

Table 5.4. Comparison of prevalence estimates of *Trypanosoma* spp. obtained using NGS and Sanger sequencing in koalas and ticks (*I. holocyclus* and *I. tasmanni*).

<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Koala</th>
<th><em>I. holocyclus</em></th>
<th><em>I. tasmanni</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NGS</td>
<td>Sanger</td>
<td>NGS</td>
</tr>
<tr>
<td><em>T. irwini</em></td>
<td>32.1</td>
<td>28.6</td>
<td>34.3</td>
</tr>
<tr>
<td></td>
<td>(25.2-39.8)</td>
<td>(21.9-36)</td>
<td>(19.1-52.2)*</td>
</tr>
<tr>
<td>Trypanosome species</td>
<td>Koala</td>
<td>I. holocyclus</td>
<td>I. tasmani</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td>Prevalence % (95%CI)</td>
<td>Prevalence % (95%CI)</td>
<td>Prevalence % (95%CI)</td>
</tr>
<tr>
<td></td>
<td>NGS</td>
<td>Sanger</td>
<td>NGS</td>
</tr>
<tr>
<td>T. gilletti</td>
<td>25 (18.7-32.3)*</td>
<td>1.2 (0.1-4.2)</td>
<td>31.4 (16.9-49.3)</td>
</tr>
<tr>
<td>T. copemani</td>
<td>27.4 (20.8-34.8)*</td>
<td>5.4 (2.5-9.9)</td>
<td>34.3 (19.1-52.2)*</td>
</tr>
<tr>
<td>T. vegrandis</td>
<td>10.1 (6.0-15.7)*</td>
<td>0 (0-2.2)</td>
<td>14.3 (4.8-30.3)</td>
</tr>
<tr>
<td>Trypanosoma sp. AB-2017</td>
<td>4.8 (2.1-9.2)</td>
<td>0 (0-2.2)</td>
<td>8.6 (1.8-23.1)</td>
</tr>
<tr>
<td>T. noyesi</td>
<td>0.6 (0-3.3)</td>
<td>0 (0-2.2)</td>
<td>0 (0-10)</td>
</tr>
</tbody>
</table>

*An asterisk indicates that the prevalence obtained by NGS is significantly higher than the prevalence obtained using Sanger sequencing (p < 0.05), for that particular trypanosome and host species.

Although the overall prevalence of *Trypanosoma* in koalas obtained using the Sanger sequencing method (35.1%, 95% CI: 27.9-42.8%) was slightly higher than the prevalence obtained by NGS (32.2%, 95% CI: 25.2-39.8%), the specific prevalences of *T. gilletti* and *T. copemani*, were significantly underestimated by Sanger sequencing when compared to results obtained using NGS (p < 0.05). Sanger sequencing also failed to identify *T. vegrandis*, *T. noyesi* and novel *Trypanosoma* sp. AB-2017 in koala blood samples.

In ticks, although the same number of positives (n = 23) was obtained by hemi-nested and single-round PCR, the overall prevalence of *Trypanosoma* sp. by NGS was slightly higher compared to that obtained using Sanger sequencing (23.1%, 95% CI: 14.9-33.1%), given that two PCR-positive
samples were not confirmed to be *Trypanosoma* DNA using the latter approach. The prevalence of *T. copemani* in ticks estimated from Sanger sequencing results was significantly lower when compared to those obtained by NGS (p < 0.05). Sanger sequencing was unable to detect *T. irwini*, *T. vegrandis* and novel *Trypanosoma* sp. AB-2017 in ticks.

In most cases of trypanosomes isolated from koala blood samples and ticks (97% and 91%, respectively), Sanger sequencing-based identifications corresponded to the most abundant species (i.e. species with greater amount of reads within the sample) identified by NGS (Appendices E and F, respectively). In addition, a total of 42 (51.2%) clean and 40 (48.8%) mixed chromatograms were obtained, which differs considerably from the proportion of single and mixed infections in both koalas and ticks, determined by NGS (Fig. 5.3). This indicates that mixed chromatograms were not necessarily obtained in all cases of trypanosome co-infections.

### 5.4.8 *Trypanosoma* infection profile of blood samples and ticks concurrently collected from the same koala host

In seven out of 15 cases in which tick and blood sample were concurrently collected from the same koala, both samples were negative for *Trypanosoma* DNA. In another seven cases, ticks removed from positive koalas were negative for *Trypanosoma* DNA. Interestingly, the trypanosome species characterized from a blood sample from one koala (*T. irwini*), was different from that of the tick (*I. tasmani*) (*T. gilletti*) collected from the same koala, as determined by Sanger sequencing. NGS analysis identified *T. irwini* (17,713 reads), *T. copemani* (18 reads), *T. gilletti* (5 reads) and *T. noyesi* (3 reads) in the koala blood sample; whereas only *T. gilletti* (33,982 reads) and *T. copemani* (7 reads) were identified in the tick.
5.5 Discussion

The application of NGS metabarcoding techniques can greatly improve our knowledge about the biodiversity of trypanosomes infecting Australian wildlife. This is the first study to successfully establish an NGS-based methodology, to audit trypanosome communities harboured by koalas and ticks. Notably the new method proved to be an efficient approach to characterise trypanosome polyparasitism, allowing identification of less abundant genotypes/species that are overlooked by Sanger sequencing alone.

The use of hemi-nested and not single-round PCR prior to NGS analysis constitutes a limitation of the present research, as nested PCR approaches involve an inherent risk of contamination and amplification biases (Park & Crowley, 2010). However, this approach was necessary to overcome the reduction in sensitivity that the addition of MiSeq adapters to the primers had on amplification efficiency, as reported in previous studies (Paparini et al., 2015a; Vermeulen et al., 2016; Paparini et al., 2017).

To date, research using NGS platforms to investigate eukaryotic parasites have mainly focused on zoonotic pathogens (Bunnik et al., 2013; Carlton et al., 2013; Grinberg et al., 2013; Hester et al., 2013; Begolo et al., 2014; Bordbar et al., 2014; Cheng et al., 2015; Leprohon et al., 2015; Paparini et al., 2015a; Zahedi et al., 2017); hence, high-throughput methods to investigate protozoan parasitic diseases in wildlife are not yet well-established (Schuster, 2008; Bik et al., 2012; Šlapeta, 2013; Vermeulen et al., 2016). More recently, an NGS pipeline was developed to analyse microbial eukaryotic communities, using Eimeria sp. as a model (Vermeulen et al., 2016). Although the method provides useful tools and insights, a broader use of an experiment designed specifically for Eimeria sp. may be problematic. This is because bioinformatics algorithms and similarity thresholds must be carefully selected and adjusted for Trypanosoma studies, relying on background
knowledge of this parasite’s phylogeny and epidemiology. The present NGS-based assay, although performed in koala blood samples and ticks, may also prove useful in future investigations of mixed trypanosome infections in a range of other invertebrate and vertebrate hosts, including domestic animals and humans. For each case, an optimisation of PCR amplification conditions may be necessary.

The reliability of the novel methodology was assessed based on several criteria. For instance, all positive controls harbouring dual or triple mixed trypanosome infections with *T. irwini*, *T. gilletti* and *T. copemani*, which had previously been identified by Sanger sequencing of cloned PCR products or species-specific PCRs (McInnes et al., 2011a; McInnes et al., 2011b), produced identical results by NGS in the present study. The assay also successfully identified the additional positive control, *T. cruzi*, which is relevant as surveillance of zoonotic pathogens in native species is a current research priority (Thompson & Thompson, 2015; Botero et al., 2016b; Cooper et al., 2017).

Phylogenetic analysis confirmed the classification of representative OTUs identified as *Trypanosoma* sp. in the pipeline, and demonstrated a high diversity between and within koala-derived trypanosomes. The analysis also indicated the phylogenetic position of the novel *Trypanosoma* sp. clade, which was strongly supported by bootstrap values and formed a sister clade to the group consisting of *T. copemani*, *T. gilletti* and *T. vegrandis*. Unfortunately, the relatively short length of amplicons, required for MiSeq sequencing, limits further inferences on the potential novel species’ genetic characterisation. Molecular analyses targeting additional loci and longer amplicons using Sanger sequencing are required for species delimitation in trypanosomes (Hamilton & Stevens, 2011). Hence, we recommend the use of NGS as the first step to investigate
trypanosome genetic diversity, which should be followed by additional Sanger sequencing of longer
amplicons for reliable phylogenetic characterization of any novel species identified.

NGS analysis of koala blood samples indicated that *T. irwini*, *T. copemani* and *T. gilletti* were the
predominant trypanosome species in this marsupial, which is in line with previous findings based
on Sanger sequencing (McInnes et al., 2011a; McInnes et al., 2011b). *Trypanosoma irwini*, besides
being the most prevalent, was also the species which produced the greatest amount of sequences
overall within most samples. Unfortunately, a critical challenge that limits interpretations related to
NGS quantitative analyses (i.e. *Trypanosoma* abundance), is that the number of sequences
generated from each variant by NGS may not reflect the number of microorganisms originally
present in the sample. This is due to PCR bias (which may skew OTUs relative abundance) and to a
possible variation in 18S rRNA gene copy numbers across the trypanosome species identified (Polz
& Cavanaugh, 1998; Reeder & Knight, 2010; Bradley et al., 2016).

*Trypanosoma vegrandis*, *T. noyesi* (identified for the first time in koalas) and the novel
*Trypanosoma* sp. AB-2017 OTU were found at a relatively low prevalence in koala hosts, and
generated considerably less sequences compared to other co-infecting variants within koala samples
such as *T. irwini*, *T. gilletti* and *T. copemani*. This may be the reason why these three species have
not yet been detected in koalas using *Trypanosoma* generic primers and Sanger sequencing. *Trypanosoma vegrandis* has only recently been isolated from koalas; and this was only possible
after morphological visualization of the parasite, which led to the use of a *T. vegrandis*-specific
assay for molecular detection (Chapter 4). However, discoveries like this may be difficult for novel
species or species with low levels of parasitaemia, which is the case of *T. noyesi* (Botero et al.,
2016b).
Further research is required to elucidate the clinical impact of Trypanosoma spp. upon koala health. In the study conducted by McInnes et al. (2011a), infections with T. irwini, T. copemani and T. gilletti were significantly associated with indicators of koala ill-health and non-survival. The genetic proximity of T. noyesi to the pathogenic T. cruzi is also of potential concern from a conservation and public health perspective, although intracellular stages of T. noyesi, suggestive of trypanosome pathogenicity, have not been observed in woylies (Botero et al., 2016b).

Importantly, prevalences of single and mixed trypanosome infections in koalas, obtained by NGS (4.8% and 27.4% respectively), were remarkably different from those obtained by cloning associated with Sanger sequencing (51.1% and 1.5% respectively) (McInnes et al., 2011b). A possible explanation is that reliance on mixed sequencing chromatograms as indicative of multiple trypanosome infections, broadly used as a selection criteria for cloning, may be inaccurate, as demonstrated by our results.

This is the first report of trypanosome polyparasitism involving up to five species. Multiple trypanosome infections involving up to 3 and 4 genotypes have been reported in koalas and woylies, respectively (McInnes et al., 2011a; McInnes et al., 2011b; Paparini et al., 2011; Botero et al., 2013; Thompson et al., 2014b). Co-infections composed of T. irwini, T. gilletti and T. copemani were the most frequent among the koala populations sampled, followed by concomitant infections with T. irwini, T. gilletti and T. copemani. Our results differ from previous observations that co-infections involving only T. irwini and T. gilletti were predominant among koalas, but are consistent with the report of T. copemani being more common as a triple co-infection with T. irwini (McInnes et al., 2009).
The implications of interactions amongst multiple trypanosomes in a single vertebrate host by either reducing or enhancing parasitaemia, virulence or pathogenicity are still unclear (Reifenberg et al., 1997; Pollitt et al., 2011; Cooper et al., 2017). A previous molecular survey identified mixed infections involving *T. vegrandis*, *T. copemani* and *T. noyesi* at a higher prevalence in a declining woylie population compared to a stable population (Botero et al., 2013). In contrast, another study suggested there may be an interspecific competition between *T. copemani* and *T. vegrandis* in woylies, whereby *T. vegrandis* may moderate the sequential establishment of *T. copemani* (Thompson et al., 2014b). Further investigations are essential to elucidate whether trypanosome polyparasitism may aggravate the consequences of infection; and to quantify the contribution of trypanosome infection, in single and mixed infections, (and co-infections with *Chlamydia* and KoRV) to the koala population decline.

NGS revealed a high trypanosome genetic diversity within *I. holocyclus* and *I. tasmani*, with five species (*T. irwini*, *T. gilletti*, *T. copemani*, *T. vegrandis* and the novel *Trypanosoma* sp. AB-2017) identified within these hosts. In contrast to NGS of koalas, in both tick species examined by NGS, *T. gilletti* was the dominant species, with the proportion of *T. irwini* sequences (up to 3.1%) being unexpectedly significantly lower than in koalas. However, although koalas and ticks investigated are from the same region of Australia, the fact that they constituted independent sampling groups (i.e. only a small proportion of ticks and blood samples were concurrently collected from the same koala), makes it difficult to make inferences about the host-vector-parasite relationships.

The differences in the trypanosome species identified in a koala blood sample and a tick removed from the same animal is interesting. NGS identified *T. irwini* in > 99.8% of sequences from the koala blood sample, whereas *T. gilletti* was the dominant species identified in the tick (which was also the dominant species in ticks for which a corresponding koala blood sample was not available).
This suggests that the trypanosome DNA detected within the tick, was unlikely to have originated from the koala blood meal. However, it is important to note that the number of sequences assigned to each *Trypanosoma* sp. by NGS does not necessarily reflect the actual parasite numbers originally present within the sample, due to possible amplification bias and because the 18S rRNA copy numbers of the different *Trypanosome* species identified in the present study (which are currently unknown) may vary. In addition, low abundance trypanosome species in the tick may still be transmitted, particularly if they are capable of multiplying/differentiating in the vector (cyclical transmission) or if able to survive in the tick’s digestive tract until it is accidentally ingested by another host (mechanical transmission). Further research on a larger number of koala blood samples and corresponding ticks and the fate of these trypanosomes in ticks is required to better understand the role of ticks in the transmission of different trypanosome species to koalas. This is the first report of trypanosome polyparasitism in Australian native ticks, which is probably due to limitations of Sanger sequencing used in previous surveys. It is possible that, in each tick species, the most prevalent and abundant *Trypanosoma* spp. is transmitted by the tick. On the other hand, it may also be possible that ticks can individually be vectors for more than one *Trypanosoma* spp. Further research is required to clarify the vectorial role of *Ixodes* ticks in *Trypanosoma* spp. transmission.

The identification of trypanosomes in an ectoparasite does not make it a vector, as the DNA detected could represent ingested parasites from a blood meal. However, mechanical transmission where the parasite can survive in the vectors’ digestive tract and then be passed on to another host cannot be ruled out (Thompson & Thompson, 2015; Cooper et al., 2017). Cross-transmission experiments from infected vector candidates (ticks) to uninfected mammalian host have previously been used to confirm vectors (Thompson & Thompson, 2015). However, this is often impractical and in the case of threatened marsupials such as the koala, it is unethical.
Based on parasite detection only, *I. australiensis* has been identified as a potential vector for *T. copemani* (Austen et al., 2011); and tabanid flies (family Tabanidae) and biting midges (family Ceratopogonidae), have been suggested as candidate vectors for *T. noyesi* (Botero et al., 2016b). In the present study, NGS of ticks identified four koala-derived *Trypanosoma* spp. within *I. tasmani* and *I. holocyclus*. To the best of our knowledge, this is the first identification of *I. tasmani* as a potential vector for *Trypanosoma* spp. A previous study relying on morphological tools only has found trypanosomes in an *I. holocyclus* tick retrieved from a bandicoot positive for *T. thylacis* (Mackerras, 1959); thus this is the first identification of *T. irwini*, *T. gilletti*, *T. copemani*, and *T. vegrandis* in *I. holocyclus*, which is based on molecular evidence.

The finding of *I. holocyclus* harbouring *T. copemani* could potentially be of public health significance, given the natural resistance of *T. copemani* to human serum (Austen et al., 2015c). Nevertheless, further research is required to determine the zoonotic potential of *T. copemani* and other koala-derived trypanosomes. From a One Health perspective, it is essential to monitor the presence of trypanosomes in wildlife and their ectoparasites using NGS, considering their potential to harbour zoonotic pathogens (Backhouse & Bolliger, 1951).

In conclusion, the novel NGS-based method for the detection and characterisation of *Trypanosoma*, described in the present study, constitutes an efficient molecular tool to determine the genetic diversity of trypanosomes in koalas and candidate vectors. Our results highlight the greater accuracy of NGS compared to Sanger sequencing, as the latter clearly underestimated the prevalence of rare isolates within the samples examined; and overlooked the presence of novel species. Therefore, Sanger sequencing of PCR amplicons obtained using *Trypanosoma* generic primers is ineffective for the detection of mixed trypanosome infections in native species.
NGS analysis provided new insights into trypanosome genetic diversity in koalas and identified, for the first time, two species of native ticks (*I. holocyclus* and *I. tasmani*) as vector candidates for *T. gilletti*, *T. irwini*, *T. copemani*, *T. vegrandis* and a novel koala-derived trypanosome. The discovery that mixed infections with up to five *Trypanosoma* spp. are significantly more prevalent than single trypanosome infections in koalas, constitutes a benchmark for future clinical and epidemiological studies required to quantify the contribution of trypanosomes to clinical disease in koalas, particularly in the presence of concurrent pathogens such as *Chlamydia* and KoRV. Such knowledge can support important decisions on koala health and population management, helping to stabilize population decline events.
Chapter 6  

Trypanosoma teixeirae: a new species belonging to the T. cruzi clade causing trypanosomiasis in an Australian little red flying fox (Pteropus scapulatus)

6.1 Preface

This chapter is a modified version of the article:

6.2 Introduction

Bats (order Chiroptera) are reservoirs of numerous zoonotic pathogens, including rabies, Australian bat lyssavirus, severe acute respiratory syndrome (SARS), Hendra virus, Nipah virus and Ebola virus (Wood et al., 2012). Numerous trypanosome species have also been identified in bats in Asia, Africa, South America and Europe (Hoare, 1972; Baker, 1973; Marinkelle, 1976, 1979; Gardner & Molyneux, 1988a, 1988b; Hamanaka & Pinto Ada, 1993; Steindel et al., 1998; Barnabe et al., 2003; Grisard et al., 2003; Lisboa et al., 2008; Cottontail et al., 2009; Maia da Silva et al., 2009; Cavazzana et al., 2010; Garcia et al., 2012; Hamilton et al., 2012; Lima et al., 2012; Lima et al., 2013; Marcili et al., 2013; Silva-Iturriza et al., 2013; Cottontail et al., 2014; Ramirez et al., 2014).

In Australia, three *Trypanosoma* spp. have been described in bats to date: *Trypanosoma pteropi* from the black flying fox (*Pteropus gouldii*) (Breinl, 1913; Mackerras, 1959), *Trypanosoma hipposideri* from the dusky horseshoe bat (*Hipposideros bicolor albanensis*) and *T. vegrandis*, in pteropid bats (Yangochiroptera) and microbats (Yinpterochiroptera) (Austen et al., 2015a). None of these have been associated with clinical disease. In addition, Mackie et al. (2017) recently described the first case of trypanosomiasis in a little red flying fox (suborder Yinpterochiroptera) from eastern Australia, caused by an apparently novel trypanosome species.

Molecular and phylogenetic studies have suggested that bat trypanosomes are implicated in the evolutionary history of the *T. cruzi* clade and may potentially be the precursor of trypanosomes from Australian marsupials and several African terrestrial mammals (Hamilton et al., 2012; Lima et al., 2013). There is however very limited knowledge about the genetic diversity of Australian bat trypanosomes, with only 9 of 76
indigenous bat species have been screened for this parasite to date (Thompson et al., 2014a).

In the present study, we describe the morphological and genetic characterisation of the novel trypanosome in the little red flying fox (Mackie et al., 2017), for which we proposed the name *Trypanosoma teixeirae*. Additionally, in the light of the discovery of a new *Trypanosoma* sp. in bats, we decided to investigate the presence of these parasites in blood samples from grey-headed flying foxes, captured at the Botanic Park, Adelaide (SA).

6.3 Materials and methods

6.3.1 Sampling

A venous blood sample was collected from the cephalic vein of an adult female little red flying fox that presented to the AZWH in April, 2014. The flying fox had been rescued from the ground at Redcliffe in south-eastern Queensland, Australia and was moribund with anaemia and icterus. Clinical and pathological evidence of disease consistent with *trypanosomiasis* in this flying fox was described by Mackie et al. (2017). Additional blood samples were collected from 87 grey-headed flying foxes captured at Botanic Park, Adelaide (SA) between September 2015 and February 2016. The sampling was conducted following standard procedures as described in chapter 2 (section 2.1).

6.3.2 Morphological analysis

Thin blood smears were made from a drop of fresh blood from the little red flying fox, stained and systematically examined by light microscopy as described in chapter 2 (sections 2.3.1 and 2.3.2). Digital light micrograph images (taken at x1,000 magnification) of any trypomastigotes observed were used to measure key morphological features according to the methods described in section 2.3.3.
As two trypanosome species have previously been described in Australian bats based on morphological analysis only (Breinl, 1913; Mackerras, 1959), morphometrics of the novel trypanosome was compared statistically with available measurements for *T. pteropi* and *T. hipposideri*. Mean values for each morphological feature were calculated for *T. teixeirae* whilst median values of reported ranges were used as input data for *T. pteropi* and *T. hipposideri*, as means were not available in the bibliographical references. Statistical analyses were conducted using the one sample t-test, in the software PAST 1.43 (Hammer et al., 2001).

### 6.3.3 Molecular analysis

DNA extractions, PCR assays and purification of PCR products were conducted as described in chapter 2 (sections 2.4.1 to 2.4.3). In this study, a nested PCR protocol using generic *Trypanosoma* sp. primers SLF, S762R, S823F and S662R (Maslov et al., 1996; McInnes et al., 2009) was performed to amplify an approximately 900bp fragment of the 18S rRNA gene, as previously described by McInnes et al. (2009). The DNA samples were also amplified at the GAPDH gene using a hemi-nested PCR protocol (McInnes et al., 2009).

The purified PCR products that corresponded to the expected product length were Sanger sequenced as described in chapter 2 (section 2.4.4). Nucleotide sequences obtained in this investigation were deposited in GenBank under the accession numbers KT907061 and KT907062.

### 6.3.4 Phylogenetic analysis

Phylogenetic relationships of nucleotide sequences obtained at both 18S rRNA and GAPDH loci with additional trypanosome sequences retrieved from GenBank (Table 6.1) were conducted as described in chapter 2 (section 2.5). In this study, the alignments
were curated by removing ambiguous regions containing gaps or poorly aligned using by Gblocks (Castresana, 2000), available on the Phylogeny.fr platform (Dereeper et al., 2008). The phylogenetic relationships at both 18S rRNA and GAPDH genes were inferred using the Tamura-Nei model (Tamura & Nei, 1993). The gamma shape parameter was estimated directly from the data. Estimates of genetic divergence between sequences were generated in MEGA 6 based on the Tamura-Nei algorithm, using uniform rates and a partial deletion of 95%.

**Table 6.1. GenBank accession numbers and sources (where known) of trypanosome isolates included in the phylogenetic analyses**

<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Host origin</th>
<th>Geographic origin</th>
<th>GenBank accession numbers</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>18S rDNA</td>
</tr>
<tr>
<td><em>T. rangeli</em></td>
<td>Bat (Platyrhinus lineatus)</td>
<td>Brazil</td>
<td>FJ900242</td>
</tr>
<tr>
<td><em>T. minasense</em></td>
<td>Tamarin (Saguinus midas)</td>
<td>Japan</td>
<td>AJ012413</td>
</tr>
<tr>
<td><em>T. dionisii</em></td>
<td>Bat (Eptesicus brasiliensis)</td>
<td>Brazil</td>
<td>FJ001666</td>
</tr>
<tr>
<td><em>T. erneyi</em></td>
<td>Bat (Tadarida sp.)</td>
<td>Mozambique</td>
<td>JN040987</td>
</tr>
<tr>
<td><em>T. vespertilionis</em></td>
<td>Bat (Pipistrellus pipistrellus)</td>
<td>England</td>
<td>AJ009166</td>
</tr>
<tr>
<td><em>T. sp. bat</em></td>
<td>Bat (Rousettus aegyptiacus)</td>
<td>Gabon</td>
<td>AJ012418</td>
</tr>
<tr>
<td><em>T. livingstonei</em></td>
<td>Bat (Rhinolophus landeri)</td>
<td>Mozambique</td>
<td>KF192979</td>
</tr>
<tr>
<td>TCC1270</td>
<td>Bat (Hipposideros caffer)</td>
<td>Mozambique</td>
<td>------</td>
</tr>
<tr>
<td><em>T. livingstonei</em></td>
<td>Bat (Myotis levis)</td>
<td>Brazil</td>
<td>FJ900241</td>
</tr>
<tr>
<td>TCC1953</td>
<td>Bat (Carollia perspicillata)</td>
<td>Brazil</td>
<td>FJ001664</td>
</tr>
<tr>
<td><em>T. cruzi</em> Tcbat</td>
<td>Human (Homo sapiens)</td>
<td>Brazil</td>
<td>AF301912</td>
</tr>
<tr>
<td><em>T. cruzi marinkellei</em></td>
<td>Rat (Rattus rattus)</td>
<td>Brazil</td>
<td>AJ012411</td>
</tr>
<tr>
<td>Trypanosome</td>
<td>Host origin</td>
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<td>GenBank accession numbers</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------------</td>
<td>-------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>T. sp. NanDoum1</td>
<td>Palm civet (Nandinia binotata)</td>
<td>Cameroon</td>
<td>FM202492 FM164793</td>
</tr>
<tr>
<td>T. sp. HochNdi1</td>
<td>Monkey (Cercopithecus nictitans)</td>
<td>Cameroon</td>
<td>FM202493 FM164794</td>
</tr>
<tr>
<td>T. noyesi H25</td>
<td>Kangaroo (Macropus giganteus)</td>
<td>Australia</td>
<td>AJ009168 AJ620276</td>
</tr>
<tr>
<td>T. noyesi AP-2011-64</td>
<td>Possum (Trichosurus vulpecular)</td>
<td>Australia</td>
<td>JN315383 AJ620276</td>
</tr>
<tr>
<td>T. noyesi AB-2013-G8</td>
<td>Woylie or brush-tailed bettong (Bettongia penicillata)</td>
<td>Australia</td>
<td>KC753537 KC812988</td>
</tr>
<tr>
<td>T. avium</td>
<td>Eagle (Aquila pomarina)</td>
<td>Slovakia</td>
<td>AF416559</td>
</tr>
<tr>
<td>T. sp. AAT</td>
<td>Currawong (Strepera sp.)</td>
<td>Australia</td>
<td>AJ620557 AJ620264</td>
</tr>
<tr>
<td>T. bennetti</td>
<td>American kestrel (Falco sparverius)</td>
<td>Germany</td>
<td>AJ223562 FJ649486</td>
</tr>
<tr>
<td>T. irwini</td>
<td>Koala (Phascolarctos cinereus)</td>
<td>Australia</td>
<td>FJ649479 FJ649485</td>
</tr>
<tr>
<td>T. lewisi</td>
<td>Rat</td>
<td>England</td>
<td>AJ009156 AJ620272</td>
</tr>
<tr>
<td>T. microti</td>
<td>Vole (Microtis agrestis)</td>
<td>England</td>
<td>AJ009158 AJ620273</td>
</tr>
<tr>
<td>T. vivax</td>
<td>Cattle</td>
<td>Europe</td>
<td>EU477537 AF053744</td>
</tr>
<tr>
<td>T. brucei brucei</td>
<td>Human</td>
<td>England</td>
<td>X59955</td>
</tr>
<tr>
<td>T. brucei rhodesiense</td>
<td>Human</td>
<td>Uganda</td>
<td>AJ009142</td>
</tr>
<tr>
<td>T. brucei gambiense</td>
<td>Human</td>
<td>Nigeria</td>
<td>AJ009141</td>
</tr>
<tr>
<td>T. evansi</td>
<td>Capybara (H. hydrochaeris)</td>
<td>Brazil</td>
<td>AJ009154 AF053743</td>
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<tr>
<td>T. copemani</td>
<td>Koala</td>
<td>Australia</td>
<td>GU966588</td>
</tr>
<tr>
<td>T. copemani G1</td>
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<td>Australia</td>
<td>KC753530 KC812982</td>
</tr>
<tr>
<td>T. copemani G2</td>
<td>Woylie</td>
<td>Australia</td>
<td>KC753531 KC812983</td>
</tr>
<tr>
<td>Trypanosome</td>
<td>Host origin</td>
<td>Geographic origin</td>
<td>GenBank accession numbers</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td><em>T. gilletti</em></td>
<td>Koala</td>
<td>Australia</td>
<td>GU966589 GU966587</td>
</tr>
<tr>
<td><em>T. vegrandis G3</em></td>
<td>Woylie</td>
<td>Australia</td>
<td>KC753533 KC812984</td>
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<tr>
<td><em>T. vegrandis G4</em></td>
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<td>Australia</td>
<td>KC753532 KC812985</td>
</tr>
<tr>
<td><em>T. vegrandis G5</em></td>
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<td>Australia</td>
<td>KC753534 KC812986</td>
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<tr>
<td><em>T. vegrandis G6</em></td>
<td>Woylie</td>
<td>Australia</td>
<td>KC753535 -----</td>
</tr>
<tr>
<td><em>T. vegrandis G7</em></td>
<td>Woylie</td>
<td>Australia</td>
<td>KC753536 KC812987</td>
</tr>
<tr>
<td><em>T. mega</em></td>
<td>African toad</td>
<td>Africa</td>
<td>AJ009157 AJ620253</td>
</tr>
<tr>
<td><em>T. rotatorium</em></td>
<td>Bullfrog (Rana</td>
<td>Canada</td>
<td>AJ009161 AJ620256</td>
</tr>
<tr>
<td><em>T. binneyi</em></td>
<td>Platypus</td>
<td>Australia</td>
<td>AJ132351 AJ620266</td>
</tr>
<tr>
<td><em>T. granulosum</em></td>
<td>Eel (Anguilla</td>
<td>Portugal</td>
<td>AJ620552 -----</td>
</tr>
<tr>
<td><em>T.sp. CLAR</em></td>
<td>Catfish (Clarias</td>
<td>Africa</td>
<td>AJ620555 AJ620251</td>
</tr>
</tbody>
</table>

### 6.4 Results

#### 6.4.1 Microscopy and morphometric analysis of *T. teixeirae*

A total of nine organisms morphologically consistent with a trypanosome, were detected by light microscopy in blood films from the little red flying fox. The extracellular organisms were slender with tapered ends, with long free flagella and either an undeveloped or absent undulating membrane. A nearly central nucleus and a terminal small round deeply staining internal structure consistent with a kinetoplast were also observed (Fig. 6.1 a, b, c). The trypomastigotes varied in length from 20.4 to 30.8 µm (average 25.9 µm) and in width from 1.3 to 2.3 µm (average 1.9 µm) (Table 6.2).
Figure 6.1. Light photomicrographs of Diff Quick stained blood film showing *T. teixeirae* trypomastigotes in the blood of a red flying fox (*P. scapulatus*) (a, b, c) and (d) Round epimastigote form. Scale bars represent 10 µm.
Table 6.2. Mean dimensions and standard errors (SE) of morphological features of *T. teixeirae* isolated from a little red flying fox’s blood

<table>
<thead>
<tr>
<th>Feature*</th>
<th>No. of organisms measured</th>
<th>Observed range (µm)</th>
<th>Mean ± SE (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length</td>
<td>8</td>
<td>20.4 - 30.8</td>
<td>25.9 ± 1.2</td>
</tr>
<tr>
<td>Width</td>
<td>8</td>
<td>1.3 - 2.3</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>PK</td>
<td>7</td>
<td>1.5 - 2.4</td>
<td>2 ± 0.15</td>
</tr>
<tr>
<td>KN</td>
<td>9</td>
<td>3.3 - 6.2</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>NA</td>
<td>9</td>
<td>5.1 - 9.8</td>
<td>7.8 ± 0.5</td>
</tr>
<tr>
<td>FF</td>
<td>8</td>
<td>10.0 - 12.9</td>
<td>11.3 ± 0.4</td>
</tr>
</tbody>
</table>

*Total length: total body length measured along mid-line including free-flagellum
Width: maximum width measured at nucleus level (undulating membrane included)
PK: distance between the posterior end and the kinetoplast
KN: distance between the kinetoplast and posterior edge of the nucleus
NA: distance between the anterior edge of the nucleus and the anterior end of the body
FF: length of the free flagellum

Among the nine long slender organisms observed, two were not true trypomastigotes as their kinetoplast was located at the very end of the posterior, which made it impossible to calculate the PK distance. In another instance, the trypomastigote’s free flagellum was apparently under a red blood cell, hence any measurements taken of FF or TL would have been inaccurate. We have therefore only measured what was feasible, which explains the divergence in the number of organisms measured for each morphological feature (Table 6.2).

Three flagellate round forms with a flagellum running around the organism about 90 degrees were also observed (Fig. 6.1d). Their body shape was consistent with a sphaeromastigote or a round epimastigote if their flagellar position was considered.

Morphometric analysis revealed that although the reported length and width ranges for the *T. teixeirae* and *T. pteropi* overlap, the former was significantly longer and thinner
than the latter \( (p < 0.01) \) (Table 6.3). There was no significant difference between KN, NA and FF dimensions between \( T. \) teixeirae and \( T. \) pteropi. In addition, \( T. \) hipposideri was significantly smaller than \( T. \) teixeirae for TL and FF dimensions \( (p < 0.01) \) (Table 6.3). No significant difference was observed for B, PK and KN between \( T. \) teixeirae and \( T. \) hipposideri.

**Table 6.3. Comparison between morphological dimensions of blood trypomastigotes of \( T. \) teixeirae with \( T. \) pteropi and \( T. \) hipposideri.**

<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Morphological Feature (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total length</td>
</tr>
<tr>
<td><em>Trypanosoma teixeirae</em> sp.n.</td>
<td>20.4-30.8</td>
</tr>
<tr>
<td></td>
<td>25.9</td>
</tr>
<tr>
<td><em>Trypanosoma pteropi</em></td>
<td>18.0-22.0</td>
</tr>
<tr>
<td></td>
<td>20.0*</td>
</tr>
<tr>
<td><em>Trypanosoma hipposideri</em></td>
<td>10.5-13.0</td>
</tr>
<tr>
<td></td>
<td>11.65*</td>
</tr>
</tbody>
</table>

Total length: total body length measured along mid-line including free-flagellum; Width: maximum width measured at nucleus level (undulating membrane included); PK: distance between the posterior end and the kinetoplast; KN: distance between the kinetoplast and posterior edge of the nucleus; NA: distance between the anterior edge of the nucleus and the anterior end of the body; FF: length of the free flagellum. Ranges given with mean for *Trypanosoma teixeirae*. As the mean dimensions were not available for *Trypanosoma pteropi* or *Trypanosoma hipposideri* the median value of the range is presented in the table and was used for statistical analysis. For each column, values followed by an asterisk are significantly different to the *T. teixeirae* value \( (p < 0.01) \).

### 6.4.2 Sequence and phylogenetic analysis

Maximum likelihood analysis of *Trypanosoma* 18S rDNA and gGAPDH sequences (Figs. 6.2 and 6.3, respectively), obtained from the little red flying fox, produced concordant tree topologies and revealed that *T. teixeirae* grouped with other trypanosomes belonging to the *T. cruzi* clade, including 7 bat-derived isolates (*T. cruzi Tcbat, T. cruzi marinkellei, T. erneyi, T. dionisii, T. rangeli, T. vespertilionis* and *T. sp. AJ012418/ GQ140365*) and three isolates from Australian marsupials (*T. noyesi H25...*
from a kangaroo- AJ009168/ AJ620276; T. noyesi AB-2003-G8 from a woylie-
KC753537/ KC812988; and T. noyesi AP-2011-64 from a common brush-tailed possum
– JN315383/ AJ620276). Similar results were obtained using the neighbour-joining
method (data not shown). The phylogenetic trees also corroborated the evolutionary
relationships among all major trypanosome clades described in previous broader
analyses.
Figure 6.2. Phylogenetic relationships of *T. teixeirae* with other trypanosomes, based on 18S rDNA partial sequences (~730bp). Evolutionary relationships were determined by maximum likelihood, based on the Tamura-Nei model (Tamura et al. 2013). Bootstrap values (> 60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site. Trypanosome species from bats are shown with an asterisk.
Figure 6.3. Phylogenetic relationships of *T. teixeirae* with other trypanosomes, based on gGAPDH partial sequences (~775bp). Evolutionary relationships were determined by maximum likelihood, based on the Tamura-Nei model (Tamura et al. 2013). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site. Trypanosome species from bats are shown with an asterisk.
Estimates of evolutionary divergence between nucleotide sequences revealed that *T. teixeirae* was genetically distinct but most closely related to *T. minasense* and *T. rangeli* (genetic distances of 1% at the 18S rDNA locus and 14%-15% at the gGAPDH, respectively) (Table 6.4).

Blood samples from grey-headed flying foxes were negative for *Trypanosoma* DNA.

Table 6.4. Genetic distances between *T. teixeirae* and other trypanosome species at 18S rRNA and gGAPDH loci.

<table>
<thead>
<tr>
<th>Trypanosome species/isolate</th>
<th>Genetic distances (%)</th>
<th>18S rDNA</th>
<th>gGAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. minasense</em> (AJ012413/ AB362561)</td>
<td>1%</td>
<td>14%</td>
<td></td>
</tr>
<tr>
<td><em>T. rangeli</em> (FJ900242/ GQ140364)</td>
<td>1%</td>
<td>15%</td>
<td></td>
</tr>
<tr>
<td><em>T. sp. bat</em> (AJ012418/ GQ140365)</td>
<td>1%</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td><em>T. conorhini</em> (AJ012411/ AJ620267)</td>
<td>1%</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td><em>T. sp. NanDoum1</em> (FM202492/ FM164793)</td>
<td>1%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td><em>T. vespertilionis</em> (AJ009166/ AJ620283)</td>
<td>1%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td><em>T. sp. HochNd1</em> (FM202493/ FM164794)</td>
<td>1%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td><em>T. noyesi</em> H25 (AJ009168/ AJ620276)</td>
<td>2%</td>
<td>17%</td>
<td></td>
</tr>
<tr>
<td><em>T. noyesi</em> AB-2013-G8 (KC753537/ KC812988)</td>
<td>1%</td>
<td>18%</td>
<td></td>
</tr>
</tbody>
</table>

Genetic distances were calculated in MEGA 6 (Tamura et al. 2013) using the Tamura-Nei model (Tamura and Nei 1993).

6.4.3 Novel species description

**Species Name:** *Trypanosoma teixeirae* (Fig. 6.1)

**Type host:** Little red flying fox (*Pteropus scapulatus*).

**Other hosts:** Unknown

**Type Locality:** Redcliffe Peninsula, Queensland, Australia.

**Prevalence:** Unknown
**Morphology:** *T. teixeirae* trypomastigotes are on average 25.9 µm long and 1.9 µm wide, exhibiting a slender shape with tapered ends, a nearly central nucleus and a small terminal kinetoplast. Undulating membrane either absent or under-developed.

**Etymology:** The species is named *T. teixeirae* in honour of Prof. Marta Maria Geraldes Teixeira, from the University of Sao Paulo, who has greatly contributed to the biology and phylogeny of trypanosome species.

### 6.5 Discussion

In the present study, we have described *T. teixeirae*, the causative agent of trypanosomiasis in an Australian little red flying fox, using morphological and molecular techniques. This is the fourth trypanosome species to be reported in indigenous Australian bats and the first one associated with clinical disease.

Unfortunately, no genetic data was available for two of the previously reported bat-derived trypanosomes in Australia (*T. pteropi* and *T. hipposideri*). *Trypanosoma pteropi* was described as having a slender body (total length 18-22 µm; width 2-4 µm), an under-developed undulating membrane and a long free flagellum whereas *T. hipposideri* is very small and slender (total length 10.5-13 µm; width 1.5-2 µm), with a delicate short free flagellum at the anterior end (Breinl, 1913; Mackerras, 1959). Statistical analysis however, revealed that *T. teixeirae* was significantly larger than both *T. pteropi* and *T. hipposideri*, even though they had several other overlapping morphometric features. However, morphology alone is not a reliable tool to delimit trypanosome species due to the interspecific similarities and intraspecific variability (Dunn et al., 1963; Marinkelle, 1966; Dunn, 1968).
Besides the typical trypomastigotes, two round forms (sphaeromastigotes or round epimastigotes) were also observed. The term ‘sphaeromastigote’ (Brack, 1968) refers to the parasite body shape only and has been applied without reference to the flagellar development. However, as these forms may occur within different stages of the parasite’s development, it is more appropriate to characterise the round organisms observed in the present study as round epimastigotes, considering both their body form and flagellar features (Elliott et al., 1974). This stage normally occurs in the interior of the cell, in vessels or in the insect gut.

Evolutionary reconstructions at both 18S rDNA and gGAPDH revealed that T. teixeirae was genetically distinct from all known trypanosomes. The use of these two genes is recommended for taxonomic analysis of trypanosomatids and validation of new trypanosome species (Hamilton et al., 2004; Viola et al., 2009; Teixeira et al., 2011; Lima et al., 2012; Borghesan et al., 2013; Lima et al., 2013).

Phylogenetic analyses at both 18S rRNA and GAPDH loci revealed that T. teixeirae clustered within the T. cruzi clade together with all other bat-derived trypanosome species described to date, except T. livingstonei (which was positioned basal to the T. cruzi clade), T. evansi (which belongs to the T. brucei clade) and T. vegrandis (which forms a separate group associated with other marsupial-derived trypanosomes found in Australia) (Hamilton et al., 2007; Botero et al., 2013; Lima et al., 2013; Austen et al., 2015a; Carnes et al., 2015). At the GAPDH locus, T. teixeirae was closest to T. minasense and T. rangeli and exhibited 14% and 15% genetic distance from these two species respectively. T. minasense has been found in neotropical non-human primates from South America (Ziccardi & Lourenco-de-Oliveira, 1999), whilst T. rangeli has been reported in a range of mammalian hosts including Brazilian bats (Maia da Silva et al., 2009). Although T. teixeirae exhibited a relatively low (1%) genetic distance from
its closest related species at the 18S rRNA locus, a similar pattern was observed when comparing other previously described species among each other. For instance, genetic distances between *T. minasense* and *T. vespertilionis* were 1% and 12% at the 18S rRNA and GAPDH loci respectively. Trypanosomes have few morphological features detectable using light microscopy which can adequately delimit species (Gibson, 2009). Previous studies have reported that a genetic distance of 3.75% at the GAPDH gene is sufficient to delimit a new trypanosome species (McInnes et al., 2011b). By this criterion, *T. teixeirae* is clearly a separate species.

Bat trypanosomes have been implicated in the evolutionary origin of *T. cruzi*, the causative agent of Chagas disease, one of the most important public health issues in South America (Hamilton et al., 2012; Bonney, 2014). The ‘bat-seeding’ hypothesis suggests that *T. cruzi* evolved from within a broad clade of bat-derived species, which have made the switch into terrestrial mammals (Hamilton et al., 2012; Lima et al., 2013). The hypothesis also implies that these arboreal trypanosomes species could potentially be evolutionary precursors for the terrestrial trypanosome lineage within Australian mammals (Hamilton et al., 2012; Lima et al., 2013; Thompson et al., 2014a). It is therefore possible that *T. teixeirae* could be the precursor of the marsupial-derived *T. noyesi* (Botero et al., 2016b), which belongs to the *T. cruzi* clade.

This study did not identify *Trypanosoma* DNA in the Australian grey-headed flying fox. Recently, a molecular investigation was also unable to detect trypanosomes in tissues of sick-euthanized grey-headed flying foxes that had been presented to Taronga Zoo (NSW) (Botero et al., 2016b). It is possible, however, that these parasites might be present in this bat species at low prevalences and parasitaemia. More extensive sampling from blood and tissues of grey-headed flying foxes and other Australian bat species are required to elucidate the epidemiology of trypanosomes in these animals.
More comprehensive baseline data on bat trypanosomes will also help to clarify the origin and evolutionary history of trypanosomes within the *T. cruzi* clade.

Similar to most bat trypanosomes described worldwide, the prevalence, distribution, vectors, life cycle and zoonotic potential of *T. teixeirae* remains unclear. Therefore, more studies comprising a larger sample size are required to better understand the prevalence and clinical impacts of *T. teixeirae* on bat populations, taking into account ecological and stress factors that could play a role in the expression of clinical disease.
Chapter 7  General discussion

The focus of this thesis was the study of prevalence and characterisation of blood-borne and enteric protozoan parasites in Australian marsupials, native rodents and bats using molecular tools and morphological analyses. The animals originated from four states/territories across northern tropical Australia, in one case by deliberate sampling of target species, whereas the others were opportunistic collections through wildlife pathologists. The surveys resulted in a significant increase in the current knowledge of the prevalence, spatial distribution, host range, taxonomy and genetic diversity of protozoan parasites in Australian wildlife.

Overall, protozoans belonging to the genus *Trypanosoma*, *Babesia*, *Hepatozoon*, *Cryptosporidium* and *Giardia* were detected in six mammal species from three states/territories in Australia: the northern brown bandicoot (NT), common brushtail possum (NT), northern quoll (NT), brush-tailed rabbit-rat (NT), koala (NSW and Qld) and little red flying fox (Qld). This research has also described a new species of *Trypanosoma* and revealed a diversity of novel protozoan parasite genotypes naturally occurring in the native fauna of Australia.

A novel NGS-based assay and bioinformatics pipeline to assess the genetic diversity of *Trypanosoma* in koalas and their ticks was developed during the present study. The results obtained highlight the benefits of using these platform technologies to effectively characterise mixed infections with different parasite species or intraspecific variants (polyparasitism) in both vertebrate and invertebrate hosts.

Another important aspect of this research was the investigation of the potential clinical impact of protozoan parasites on their native hosts. This was analysed by correlating clinical, haematological and biochemical parameters (when available) with the presence
or absence of infection. Northern brown bandicoots from the NT, that were positive for *Trypanosoma* sp. exhibited a significantly lower PCV compared to negative bandicoots (p = 0.046). We also report for the first time a trypanosome infection associated with clinical disease in bats.

### 7.1 Molecular and morphological methods for the detection and characterisation of protozoan parasites

In this thesis, both traditional molecular approaches and high throughput DNA sequencing were employed to provide reliable estimates of the prevalence of blood-borne and enteric protozoan parasites in Australian native mammals. Moreover, phylogenetic analyses were conducted to shed light into evolutionary relationships and determine the extent of genetic diversity between and within parasite species.

In addition to providing sensitive identification of known parasite taxa, the application of molecular tools in the present study has resulted in the description of a novel parasite species (*T. teixeirae*) in an Australian little red flying fox. *Trypanosoma teixeirae* is the fourth trypanosome species to be reported in indigenous Australian bats. Future studies comprising a larger sample size must be conducted to determine the prevalence, host range, biogeography and pathogenicity of *T. teixeirae*. The present study also resulted in the identification of several novel *Trypanosoma*, *Babesia* and *Hepatozoon* genotypes from a range of marsupial species. Furthermore, DNA sequences and subsequent phylogenetic analysis of *Giardia peramelis* at the *gdh* locus helped to further clarify its phylogenetic position, supporting its species status.

Amongst the molecular methods performed during this investigation were single-round, nested or hemi-nested generic PCR’s followed by direct Sanger sequencing of the resulting amplicons. Overall, blood samples were tested at the 18S rRNA locus for trypanosomes, piroplasms and haemogregarines, with faecal samples screened for
Cryptosporidium spp. at the 18S rRNA locus, and for Giardia spp. at the gdh and 18S rRNA loci. For species description purposes, blood samples from the little red flying fox were screened at an additional locus (gGAPDH), as reliance on 18S rRNA data alone is not sufficient for validation of a new trypanosomatid species (Hamilton et al., 2004; Viola et al., 2009; Teixeira et al., 2011; Lima et al., 2012; Borghesan et al., 2013; Lima et al., 2013).

Two novel molecular techniques were developed during the present research to identify genetic variants involved in Trypanosoma poly parasitism. Poly parasitism, which refers to concurrent infections by either different species of parasites, and/or intra-specific variants of the same species of parasite (Lymbery & Thompson, 2012), seems to be the rule rather than the exception in nature (de Castro & Bolker, 2005), and the possibility of either synergistic or antagonistic effects between them must be considered.

The first method was developed to confirm, for the first time, the presence of T. vegrandis in koalas, after small flagellate organisms consistent with the morphological description of T. vegrandis (Thompson et al., 2013), were observed in blood smears from koalas. The successful technique consisted of using 1 µl of purified gel product (and not primary PCR mixture) from the primary PCR (McInnes et al., 2009), as a DNA template in the internal species-specific reaction (Botero et al., 2013). This alternative proved useful to isolate T. vegrandis sequences that were probably present in relatively lower amounts within complex DNA mixtures. This approach was necessary after attempts to detect T. vegrandis in a koala blood sample (that was positive for T. vegrandis by microscopy), using generic primers and a previously described T. vegrandis-specific assay (Botero et al., 2013), failed to amplify T. vegrandis, but instead unexpectedly amplified T. gilletti from that sample. Our hypothesis is that T. vegrandis had not previously been identified in koalas due to its small size and the fact that
generic PCR and Sanger sequencing may mask the presence of less abundant genotypes. Furthermore, the use of a range of species-specific primer sets in a previous molecular investigation of trypanosomes in koalas (McInnes et al., 2011a), although useful to resolve mixed infections, was limited to known species only (at the time, only *T. irwini*, *T. gilletti* and *T. copemani* were known to infect koalas).

The finding of mixed infections involving *T. vegrandis* and up to two more trypanosome species (*T. gilletti* and/or *T. irwini*) in koalas, during the present study, supports previous evidence that mixed trypanosome infections are common in koalas (McInnes et al., 2011a; McInnes et al., 2011b). This also prompted the need for a more convenient, robust and accurate method, based on NGS of amplicons, to effectively characterise mixed trypanosome infections in koalas. NGS offers the advantage of massive parallelization of sequencing reactions and, combined with bioinformatics, has revolutionised the research field of molecular epidemiology and diagnosis of infectious diseases. The method has effectively overcome the limitations of conventional techniques (e.g. the issue of culture bias or culture fitness between strains of the same species, bias imposed by the use of species-specific PCR to known species only and the time-consuming nature of screening large numbers of samples using conventional PCR and Sanger sequencing). The NGS-based approach not only confirmed the presence of *T. vegrandis* in koalas and ticks removed from koala hosts, but also uncovered a number of genotypes belonging to a potential novel species (overlooked by Sanger sequencing). Importantly, the NGS assay allowed for the discovery that mixed trypanosome infections with up to five different trypanosome species are significantly more prevalent than single trypanosome infections in koalas (p < 0.05).

Overall, most of the blood-borne parasites detected by PCR were not observed in corresponding blood smears by light microscopy. This finding is now well recognised
and understood, that molecular approaches are more sensitive than microscopy for protozoan parasite detection, particularly in cases of low parasitaemia (Morgan et al., 1998; Wicks et al., 2006; Pizarro et al., 2007; Tavares et al., 2011; Botero et al., 2016b).

Although microscopy is an invaluable tool in terms of morphometric characterisation of parasites, study of life-cycle stages and determination of parasitaemia, the taxonomy of protozoa cannot be delineated based on morphometric descriptions alone. The increasing use of molecular methods has revealed that neither individual morphotypes nor their combination within a given life-cycle hold any taxonomic value, as they are randomly distributed in sequence-based phylogenetic trees (O'Donoghue, 2017). Thus, protozoan parasites may exhibit high inter- and intraspecific variability in terms of morphology, which may not be reflected in their genetic sequences (Hoare, 1972; Gibson, 2009; Ryan & Power, 2012). For example, recent studies have shown that Australian trypanosomes are highly pleomorphic, yet their genetic sequence are less diverse (Thompson et al., 2013; Austen et al., 2015b; Botero et al., 2016a).

Nonetheless, morphological analysis was crucial for the characterisation of the novel trypanosome species, *T. teixeirae* in the bat, as genetic data was not available for *T. pteropi* and *T. hipposideri*, the two species previously recorded in Australian bats (Mackerras, 1959). Microscopy was also particularly useful for the identification of *T. veyrandis* in koalas, as the initial microscopic identification of small trypomastigotes led to the subsequent molecular investigation.

### 7.2 Prevalence, host range and biogeography of blood-borne and enteric protozoan parasites in Australian native mammals

This thesis provides valuable additional baseline prevalence data and evidence of a broader host range and geographic distribution of protozoan parasites from the genera
Trypanosoma, Babesia, Hepatozoon, Cryptosporidium and Giardia in Australian marsupials, rodents and bats. The present study involved screening a total of 509 blood and 167 faecal samples from animals belonging to seven species (northern brown bandicoots, common brush-tailed possums, northern quolls, brush-tailed rabbit-rats, koalas, a little red flying-fox and grey-headed flying foxes), which were distributed across four states/territories in Australia (NT, Qld, NSW and SA).

The overall prevalence of blood-borne protozoan parasites in native mammals was 22.8% (116/509; 95% CI: 19.2 - 26.7%), whereas the prevalence of enteric parasites was only 3% (5/167; 95% CI: 1.0 – 6.8%). Protozoan parasites were identified in all species examined, except for northern quolls from the NT and grey-headed flying foxes from SA. Interestingly, given previous reports in Australian native mammals, Theileria spp. were not identified in any of the animals tested. A robust prevalence estimate of protozoan parasites in wildlife, particularly rare species, is difficult to determine given the limitations in sample sizes. In addition, many populations across the Australian mainland and islands have yet to be examined (Thompson et al., 2014a), therefore the current knowledge on the biogeography of Australian protozoan parasites is still far from complete.

Overall, in the present study, trypanosomes were detected at a relatively high prevalence amongst the animal populations studied. High prevalences of Trypanosoma spp. have also been previously reported in Australian native mammals (McInnes et al., 2011a; McInnes et al., 2011b; Paparini et al., 2014; Thompson et al., 2014a; Austen et al., 2015a). The implications of high or low prevalence of each parasite in their host species, in either single or mixed infections, warrants further investigation. However, the baseline prevalence data presented in the present thesis provides important insights into parasite diversity and distribution in Australia, and the potential adverse effects that
these organisms may have on the health of their wildlife hosts. This is of particular concern in animals that have experienced alarming population declines (Rhodes et al., 2011; Woinarski et al., 2015; Ziembicki et al., 2015) and are exposed to additional or increased levels of stress due to, for example, human encroachment, climate change and other infectious diseases (Wyatt et al., 2008; Rhodes et al., 2011).

The data presented in the present thesis extends the host range of *T. vegrandis* to include koalas and northern brown bandicoots, and provides the first identification of this parasite species in the NT. Similarly, *T. noyesi* was identified for the first time in koalas and common brush-tailed possums from the NT. These observations support previous evidence that *T. vegrandis* and *T. noyesi* are not host-specific and exhibit a widespread geographical distribution in Australia (Paparini et al., 2011; Botero et al., 2013; Austen et al., 2015a; Botero et al., 2016b; Cooper et al., 2017). This lack of host specificity in Australian trypanosomes may have important consequences for the evolutionary and ecological interactions between trypanosomes and their marsupial hosts (Thompson et al., 2014a).

Several novel *Trypanosoma* genotypes closely related to other marsupial-derived species (e.g. *T. gilletti, T. copemani* and *T. vegrandis*), were also identified in northern brown bandicoots and koalas at relatively low prevalences. Additional population studies are required to characterise these potential novel species in terms of prevalence, evolutionary relationships, host and spatial distribution, transmission dynamics and pathogenicity.

The molecular survey conducted in NT mammals revealed the presence of a novel *Babesia* genotype and several novel *Hepatozoon* genotypes in northern brown bandicoots, both at a prevalence of 9.7% (95% CI: 2.7-9.3%). The true prevalence of
piroplasms in Australian native mammals is unknown, although previous studies have identified these parasites in small studies of mammal populations from geographically disparate regions (Mackerras, 1959; O’Donoghue & Adlard, 2000; Clark et al., 2004; Clark & Spencer, 2007; Barker et al., 2012; Paparini et al., 2012b; Rong et al., 2012; Kessell et al., 2014; Donahoe et al., 2015; Šlapeta et al., 2017). This is the second report of a piroplasm in a native mammal from the NT. *Babesia thylacis* has been recorded in a northern brown bandicoot from Qld, using morphological methods only (Mackerras, 1959). The *Hepatozoon* genotypes found in northern brown bandicoots are likely to be *H. peramelis*, as morphometric features of the gametocytes observed were consistent with those belonging to that species. Unfortunately no DNA sequences are available for *H. peramelis* (Welsh & Dalyell, 1909; Mackerras, 1959). If this is the case, this represents additional evidence that *H. peramelis* has a broad distribution in Australia, occurring in NSW, Qld, Tasmania, WA and NT (Welsh & Dalyell, 1909; Mackerras, 1959; Bettiol et al., 1996; Wicks et al., 2006).

The presence of *Cryptosporidium* sp. isolate BTP1 was also reported for the first time in a common brush-tailed possum from the NT. This parasite variant has been previously reported in possums from NSW (Hill et al., 2008). This identification of BTP1 in possums from the NT corroborates the hypothesis that *Cryptosporidium* sp. BTP1 may be host-specific and provides evidence of its wider geographic distribution. Further studies comprising larger sample sizes are required to determine the prevalence and biogeography of *Cryptosporidium* sp. BTP1 in Australia.

The identification of *Giardia* sp. in mammals from the NT is consistent with previous assumptions that *Giardia* spp. are ubiquitous enteric protozoan parasites of vertebrates (Thompson, 2004; Thompson & Ash, 2016). In addition, the low prevalence of *Giardia* sp. (2.4%; 95% CI: 0.7-6.0%) in the animals tested in the present study, is in line with
the previous observation that this parasite is remarkably rare in native wildlife in WA (Thompson et al., 2010). Importantly, the present results reveal that *G. peramelis* is not host-specific as previously speculated (Adams et al., 2004; Thompson et al., 2010; Hillman et al., 2016), as molecular evidence supports its host range extension to include the common brush-tailed possum, northern brown bandicoot and brush-tailed rabbit rat.

### 7.3 Phylogeny and genetic diversity of blood-borne and enteric protozoan parasites in Australian native mammals

Overall, this research provides increased knowledge about the phylogeny and genetic diversity of protozoan parasites harboured by Australian native mammals. In particular, the results provide further evidence that Australian trypanosomes have a high inter- and intra-specific genetic diversity. The impact of high levels of parasite genetic variation on transmission, infectivity and virulence warrants further investigation.

In the present study, NGS and bioinformatics analyses allowed for a robust estimate of the natural genetic diversity of *Trypanosoma* communities infecting koalas, providing the molecular identification of six distinct species represented by at least 14 novel genotypes. Phylogenetic analyses and estimates of evolutionary distances revealed that most OTUs (99.6%), which together represented the majority (99.8%) of the sequences obtained, corresponded to known taxa. These OTUs shared between 97% and 100% genetic similarity at the 18S rRNA locus with reference sequences from *T. irwini*, *T. copemani*, *T. gilletti*, *T. vegrandis* and *T. noyesi*. Due to the relatively short length of amplicons required for sequencing on the MiSeq platform, which precludes conclusive phylogenetic analysis, Sanger sequencing of longer amplicons and additional loci are required to determine the species status of the novel clade of trypanosomes identified in koalas by NGS.
The phylogenetic position of the newly reported *T. noyesi* genotypes from possums and koalas is in line with previous studies showing intraspecific diversity within this trypanosome species (Noyes et al., 1999; Stevens et al., 1999; Hamilton et al., 2007; Paparini et al., 2011; Botero et al., 2013; Botero et al., 2016b). Likewise, high intraspecific genetic variation was observed amongst all *T. vegrandis* genotypes. According to Cooper et al. (2017) and the results presented through this thesis, it is not clear whether the different genotypes that exist in the *T. vegrandis* clade represent distinct species as the clade exhibits high levels of intraspecific diversity. The taxonomic and epidemiological significance of this is yet to be resolved.

This study provides additional evidence that some Australian trypanosomes (e.g. *T. teixeirae, T. noyesi* and *T. irwini*) are genetically more closely related to exotic species such as *T. cruzi* than to other native species (McInnes et al., 2009; Paparini et al., 2011). The implications of the genetic proximity of Australian trypanosomes and *T. cruzi* are unclear and warrant further investigation given the medical importance of *T. cruzi* as the causative agent of Chagas’ disease in South America. Interestingly, in terms of evolution, the phylogenetic position of *T. teixeirae* supports the ‘bat-seeding’ hypothesis (Hamilton et al., 2012), which suggests that bat trypanosomes may be the precursors of trypanosomes from Australian marsupials and of several African terrestrial mammals. As most bat populations in Australia remain understudied, future research is required to provide more evidence to support the ‘bat-seeding’ hypothesis in Australia and further elucidate evolutionary relationships between trypanosomes.

The novel *Hepatozoon* genotypes from northern brown bandicoots clustered with Australian *Hepatozoon* sequences from southern brown bandicoots from WA (Wicks et al., 2006). Although at least 12 distinct genotypes are now described from *Hepatozoon* species from Australian marsupials (as shown in the phylogenetic analyses conducted
during this thesis), the relatively small genetic divergence among these genotypes (up to 0.4%) suggest they are all variants of the same un-named species. More research is also required to further elucidate the genetic diversity of enteric protozoan parasite populations in Australian mammals. Such studies should also attempt to include morphological and epidemiological descriptions as well as molecular analyses to help resolve taxonomic issues.

7.4 Clinical impact of blood-borne and enteric parasites on Australian native mammals

Although the majority of wildlife trypanosomes have historically been considered benign in their wildlife hosts (Hoare, 1972; Thompson et al., 2014a), clinical evidence provided in the present study is in agreement with previous reports that trypanosomes may adversely affect the health of their native hosts in Australia, which are already threatened by wider ecosystem changes (McInnes et al., 2011a; Botero et al., 2013; Thompson et al., 2014b; Austen et al., 2015b; Hing et al., 2016).

In the present research, no evidence of major clinical disease associated with protozoan parasitic infections was observed; however, northern brown bandicoots positive for *Trypanosoma* exhibited a significantly lower PCV, compared to negative bandicoots \( p = 0.046 \). This finding highlights the potential subtle effects some trypanosomes may be having on their hosts, as also reported by McInnes et al. (2011a) and Austen et al. (2015b).

The lack of major clinical signs of disease alone is not sufficient to rule out parasitic infections as contributing to the decline events of native mammals in the NT. For instance, in addition to causing anaemia, chronic effects of trypanosomes such as immunosuppression, muscle inflammation and necrosis are often undetectable during
clinical assessments and could result in reduced fitness and therefore increasing predisposition to predation (Botero et al., 2013; Thompson et al., 2014b; Botero et al., 2016a).

Similarly, the absence of clinical signs of disease associated with piroplasms and enteric protozoans, although indicative of a stable host-parasite relationship, does not exclude these parasites as one of the factors contributing to population declines. For example, the removal of parasitized and therefore compromised individuals from the population (e.g. by predation or other secondary mechanisms) could bias the results of prevalence surveys towards individuals with greater immunocompetence (and hence lower parasitism). Longitudinal studies comprising large sample sizes are required to ascertain the role protozoan parasites play in the population decline of native mammals from the NT.

This thesis also reports the first case of clinical trypanosomiasis in an Australian bat, which was associated with *T. teixeirae*. The fact that only one sample was available for analysis hinders further assumptions with regards to the pathogenicity of *T. teixeirae* in bat populations. The two most likely causes for the major illness and post-mortem findings associated with *T. teixeirae* infection are: (1) *T. teixeirae* is highly virulent and therefore negatively affected the health of the host through direct pathogenic mechanisms; or (2) the novel species induced pathogenic effects because this particular host was immunocompromised. The possibility of trypanosome-induced immunosuppression leading to increased susceptibility to the effects of concurrent pathogens should also be considered in future research. An increased sampling depth will be required in future studies in order to derive a true understanding of the clinical impact of *T. teixeirae* on Australian bats and other potential vertebrate hosts.
Unfortunately, clinical and haematological data from the koalas examined in the present study was not available. However, the report of a significantly high prevalence of trypanosome polyparasitism in koalas from eastern Australia raised important research questions. These include whether and how mixed trypanosome infections are adversely affecting the health and welfare of koala hosts, particularly in the presence of other highly prevalent pathogens such as *Chlamydia* and KoRV. According to a report by Ezeamama et al. (2008), whilst individual pathogen infections may be tolerated and only present modest clinical signs of disease, interaction effects among multiple pathogens may dramatically alter their pathogenicity. Hence, mixed infections have been increasingly recognized as a conservation challenge, as they can result in disease-induced declines and extinctions (de Castro & Bolker, 2005; Joseph et al., 2013).

### 7.5 Potential vectors and transmission of trypanosomes

The present study reports, for the first time, the molecular detection of *Trypanosoma* spp. in two native tick species: *I. holocyclus* and *I. tasmani*. This is also the first record of trypanosome polyparasitism in invertebrate hosts in Australia. This discovery was possible using NGS-based analysis of *Trypanosoma*. It is likely that previous research failed to detect mixed trypanosome infections within potential vectors due to the limitations of morphological analysis (since Australian trypanosomes are often present in low parasitaemia and are highly polymorphic) and/or direct Sanger sequencing of resulting amplicons from *Trypanosoma*-generic PCR (Mackerras, 1959; Hamilton et al., 2005; Austen et al., 2011; Paparini et al., 2014; Botero et al., 2016b).

Further research is required to clarify the vectorial role of *I. holocyclus* and *I. tasmani*, as the *Trypanosoma* DNA detected in engorged ticks could merely represent ingested parasites from a blood meal. Future studies should associate molecular analyses with tick dissections and morphological characterisation of trypanosome life-cycle stages.
within the ectoparasite, at different time points (Austen et al., 2011). Although experimental transmission is the ultimate requirement to confirm the role of an ectoparasite as a disease vector, this method would be unethical in the case of threatened fauna.

Additional investigation is also required to determine the practical implications of trypanosome polyparasitism in ticks including which (if any) of the various trypanosome species ingested during a blood meal are able to multiply within the tick (cyclical transmission) or survive in the tick’s guts until it is accidentally ingested by another host (mechanical transmission).

The identification of trypanosome vectors is a current research priority in Australia, from the point of view of wildlife conservation and One Health (Thompson & Thompson, 2015). It will allow for an increased knowledge about the life-cycles, transmission routes and epidemiology of native Australian trypanosomes, resulting in more targeted management and conservation strategies of marsupial hosts in the future. For instance, re-introduction of naïve animals will be avoided into areas where both the vectors and pathogens are known to be present.

### 7.6 One Health implications of blood-borne and enteric parasites in Australian native mammals

No protozoan pathogens known to infect humans were identified in the native mammals and ticks examined during the present study. However, the zoonotic potential of the parasite species identified still requires further investigation. A recent study has demonstrated that *T. copemani* is resistant to human serum *in vitro* and therefore may have the potential to be infective for people (Austen et al., 2015c). Furthermore, it has been demonstrated that *T. copemani* is able to invade cells in a manner similar to the zoonotic pathogen *T. cruzi* (Botero et al., 2013). The fact that a high prevalence of *T.*
*copemani* (34.3%) was detected in human-biting ticks (*I. holocyclus*) in the present work, increases the need of determining the zoonotic potential of this parasite. Another interesting hypothesis that requires attention is that, due to the close genetic similarity between *T. noyesi* and *T. cruzi*, the vector(s) of *T. noyesi* could potentially be able to transmit *T. cruzi* from infected immigrants to native wildlife, and therefore establish the cycle of infection in Australia (Thompson & Thompson, 2015). The risks that *T. teixeirae* may pose to wildlife, livestock and human health should also be explored in future studies.

The novel NGS-based assay for *Trypanosoma* developed in the present research may assist surveillance of Australian borders for exotic pathogens to livestock and humans such as *T. evansi* and *T. cruzi*, respectively. This is because the method has the potential to uncover rare species present in low abundance within samples harbouring mixed infections, and is applicable for screening a range of vertebrate and invertebrate hosts. *Trypanosoma evansi* is the causative agent of surra, which is responsible for considerable livestock production losses in endemic countries and is considered a high quarantine risk for Australia’s northern borders (Reid, 2002). Importantly, experimental evidence suggests that surra could also cause acute disease with high mortality in marsupials (Reid, 2002).

Although no zoonotic genotypes of *Cryptosporidium* and *Giardia* were reported in this study, continuous surveillance is imperative as marsupials are the dominant animals inhabiting many water catchment areas in Australia and are known to play a role in the dissemination of human-infectious waterborne pathogens to drinking water sources (Zahedi et al., 2016a; Zahedi et al., 2016b).
7.7 Management implications and control of blood-borne and enteric parasites in Australian native mammals

There is an increasing concern that infection with protozoan parasites in Australian wildlife may be an overlooked threatening process (Thompson et al., 2014a; Cooper et al., 2017). Molecular epidemiological studies such as the present one, involving robust and reliable detection of protozoan parasites, are crucial to inform government and foster the development of superior surveillance systems to preserve wildlife populations and control zoonotic diseases in Australia. Furthermore, accurate epidemiological information aids in the prioritization of limited resources to threat mitigation and wildlife conservation and welfare.

In this context, the knowledge provided in the present thesis may be incorporated into zoonotic disease management strategies, wildlife translocation programs and threat abatement plans to sustain threatened native mammal species in Australia. The information is also useful for wildlife veterinarians who may consider the epidemiological aspects and potential clinical impact of blood and waterborne pathogens in their clinical assessments. Nevertheless, besides baseline information on prevalence and genetic/morphological diversity, an effective control of pathogenic protozoans also requires scientific knowledge on the life cycles, transmission dynamics and vectors. These aspects are an important research gap in terms of Australian protozoan parasites.

7.8 Conclusions

This study constitutes an important step towards an improved understanding of the prevalence, genetic diversity and potential clinical impact of blood-borne and enteric protozoan parasites on Australian native mammals. Further, novel information is reported on the prevalence and genetic diversity of mixed trypanosome infections in
koalas and two species of native ticks. Molecular and morphological methods were used to achieve the aims of this thesis, including a novel through-put diagnostic and surveillance method for Trypanosoma, which was crucial for a better understanding of trypanosome polyparasitism in koalas and ticks. The conclusions of this research are:

- Trypanosomes are prevalent in common brush-tailed possums and northern brown bandicoots from the NT. Infections with Babesia sp. and Hepatozoon sp. are present in bandicoots from the same territory.
- Trypanosoma, Babesia and Hepatozoon spp. identified in Australian mammals have a widespread distribution, low levels of host-specificity and high inter and intra-species genetic diversity;
- Cryptosporidium spp. and Giardia spp. are rare in small and medium-sized native mammals from the NT, and these animals do not seem to play a role in the transmission of zoonotic Cryptosporidium and Giardia genotypes;
- Trypanosoma vegrandidis and a novel Trypanosoma sp. are associated with anaemia in northern brown bandicoots and therefore may represent a threat to this marsupial species;
- Parasitic infections with T. vegrandidis are prevalent in koalas from Qld and NSW;
- NGS is an effective technique to audit trypanosome communities in blood samples from koalas and ticks removed from koala hosts;
- Co-infections with up to five trypanosome species in koalas are significantly more prevalent than single trypanosome infections. Understanding the clinical impact of such mixed infections, in the presence of other known concurrent pathogens such as Chlamydia and KoRV, is imperative to help conserving wild koala populations.
- Two species of Ixodes ticks (I. holocyclus and I tasmani) constitute potential vectors for Trypanosoma;
The trypanosome associated with acute signs of trypanosomiasis in a little red flying fox (Mackie et al., 2017) is a novel species that clusters within the *T. cruzi* clade. Its phylogenetic position further supports the previously stated hypothesis that trypanosomes from terrestrial mammals evolved from bat-derived species;

- Trypanosomes were not detected in the limited number of grey-headed flying foxes screened from SA, however it is possible that these parasites might be present in this bat species at low prevalences and parasitaemia.

- Zoonotic genotypes of trypanosomes, piroplasms, *Cryptosporidium* and *Giardia* were not present in the animals investigated; however, further studies are required to determine the role of native mammals as potential reservoirs for zoonotic protozoan pathogens in Australia.

### 7.9 Future Research

While the newly gained knowledge presented in this thesis has greatly increased our understanding about the prevalence, host-range, biogeography and genetic diversity of Australian protozoan parasites, further screening for these organisms remains a major research requirement as most wildlife populations across Australia remain un-sampled.

The characterisation of the development cycle, vectors and transmission routes of native Australian trypanosomes is another issue that requires urgent attention. Additionally, clinical, epidemiological and cell culture-based studies are essential to determine the pathogenicity and virulence of different genetic variants, as well as their potential infectivity for humans.

Studies on the transmission dynamics of protozoan parasites are hampered by the lack of suitable subtyping tools. More variable genetic loci need to be identified and used in Multi Locus Sequence Typing (MLST) to provide a better understanding of protozoan
population genetics, geographic distribution and parasite tracking. Whole genome sequencing is essential to identify these loci, as well as providing information on more complex traits of epidemiological significance, such as virulence and drug susceptibility. Complete genome analysis will also allow for more comprehensive and robust phylogenetic analyses in the future.

Further analyses of the spatial and temporal dynamics of protozoan parasites in the NT are important in order for us to determine the role that these organisms might play in the declines of native mammal populations observed in northern Australia. Ongoing molecular surveillance of protozoan parasites in wildlife is also recommended to support rapid and targeted mitigation strategies for exotic pathogens that could enter Australia and have devastating effects on immunologically naïve populations.

Lastly, an important knowledge gap is the role that mixed pathogen infections have on koala survival and fertility. This should be addressed in future studies employing NGS for complete blood and faecal pathogen profiling in wild koala populations, by aligning these data with clinical and pathological parameters. Integrated population modelling may also inform on the role of mixed infections on koala population dynamics and their implications for management and conservation.
References Cited


nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt b) genes of trypanosomes of the subgenus *Schizotrypanum* parasitic in Brazilian bats. *International Journal for Parasitology, 40*(3), 345-355.


McInnes, L. M., Hanger, J., Simmons, G., Reid, S. A. & Ryan, U. M. (2011b). Novel trypanosome *Trypanosoma gilletti* sp. (Euglenozoa: Trypanosomatidae) and the extension of the host range of *Trypanosoma copemani* to include the koala (*Phascolarctos cinereus*). *Parasitology, 138*(1), 59-70.


validation of the genera *Angomonas* and *Strigomonas* of trypanosomatids harboring bacterial endosymbionts with the description of new species of trypanosomatids and of proteobacterial symbionts. *Protist, 162*, 503–524.


a species recovery programme. *International Journal for Parasitology: Parasites and Wildlife, 4*(2), 190-196.


Appendices


Appendix E: Taxonomic characterisation of trypanosome-specific sequences obtained from koalas, by NGS and Sanger sequencing at the 18S rRNA locus. Values represent the number of sequences obtained by NGS, from each sample. Genbank codes correspond to the sequences’ closest matches.
Appendix F: Taxonomic characterisation of trypanosome-specific sequences obtained from ticks, by NGS and Sanger sequencing at the 18S rRNA locus. Values represent the number of sequences obtained by NGS, from each sample. Genbank codes correspond to the sequences’ closest matches.


Chapter 24

Haemoprotozoan parasites

Amanda Barbosa, Peter Irwin, Una Ryan

Haemoprotozoan parasites are unicellular eukaryotic organisms found in the blood of vertebrate hosts. Infections range from asymptomatic to fatal, depending on the parasite species' virulence and the immunological status of the host. The advent of molecular techniques has provided an opportunity to focus research on the biodiversity of haemoprotozoan parasites infecting Australian native mammals. Despite this, relatively little is known about the epidemiology, life cycles, transmission dynamics and clinical significance of Australian indigenous haemoprotozoa.

Haemoprotozoa of the Order Trypanosomatida and Piroplasamida are not only widespread but also appear to have the greatest potential to adversely affect the health of Australian native fauna and may be associated with population declines of some marsupial and murid species. Subclinical infections may represent an additional threat to native mammals that may be under stress from other threatening processes such as habitat degradation and human encroachment.

This chapter provides a compilation of the current knowledge of haemoprotozoan parasites documented in Australian mammals, particularly trypanosomes and piroplasms of the genus Babesia and Theileria.

1. Trypanosomes

Trypanosomes are flagellated protozoan parasites which generally have a diphasic life cycle occurring between a vertebrate host and an invertebrate vector. Whilst some trypanosome species can cause serious human diseases such as African and American trypanosomiasis (sleeping sickness and Chagas disease respectively), the majority of wildlife trypanosomes have historically been considered benign in their vertebrate hosts (Hoare 1972; Thompson et al. 2014a).

The basic morphology of mammalian trypanosomes is somewhat lanceolate and oval in transverse section; containing a kinetoplast (an extranuclear mass of DNA near the posterior end) and a single undulating flagellum at the anterior end (Fig. 24.1 and 24.2). Recent studies have demonstrated great morphological diversity within and between trypanosome species in Australia, with reported average lengths ranging from 8.3 μm to 38 μm, and average widths ranging from 1.3 μm to 15 μm (Austen et al. 2009; McInnes et al. 2009; Thompson et al. 2013; Austen et al. 2015a; Cooper et al. 2016).
Figure 24.1: *Trypanosoma copemani* isolated from the blood of a Gilbert’s potoroo (*Potorous gilberti*) a) Light micrograph, scale bar represent 10 µm. b) Scanning electron micrograph of *T. copemani* from *in vitro* culture originally isolated from a Gilbert’s potoroo. Images: Dr. Jill Austen.

Figure 24.2: Light micrograph of *Trypanosoma teixeirae* isolated from the blood of a little red flying-fox (*Pteropus scapulatus*). Scale bars = 10 µm.

Australian trypanosomes also exhibit high intra and inter-species genetic diversity. Interestingly, phylogenetic studies have demonstrated that some indigenous species are more closely related to species outside Australia. For instance, *Trypanosoma noyesi* and *T. teixeirae* are genetically more
similar to *T. cruzi* and bat-derived trypanosomes from South America and Africa, than to other Australian native marsupial-derived trypanosomes, such as *T. irwini, T. copemani, T. gilletti* and *T. vegrandis* (Hamilton *et al.* 2004; Hamilton *et al.* 2012; Thompson *et al.* 2014a; Barbosa *et al.* 2016a; Cooper *et al.* 2016).

In total, 69 native Australian mammal species have been screened for trypanosomes to date; including 46 of the 162 marsupial species (28%), 10 of the 66 rodent species (15%), 11 of the 76 bat species (14%) and both monotreme species (100%) (Thompson *et al.* 2014a; Austen *et al.* 2015b; Barbosa *et al.* 2017a). From these, 10 *Trypanosoma* spp. have been taxonomically described and eight genotypes reported (Table 24.1).

Table 24.1: Trypanosomes of native Australian mammals
<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Host (s)</th>
<th>Source/distribution</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trypanosoma binneyi</em> 2,3,5,6,8,9,18</td>
<td>Platypus (<em>Ornithorhynchus anatinus</em>)</td>
<td>Tas.</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. irwinii</em> 13-15,27</td>
<td>• Koala (<em>Phascolarctos cinereus</em>)</td>
<td>NSW, Qld, ACT</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>• Eastern bettong (<em>Bettongia gaimardi</em>)</td>
<td></td>
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<tr>
<td></td>
<td>• Brush-tailed rock-wallaby (<em>Petrogale penicillata</em>)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>• Yellow-footed rock-wallaby (<em>P. xanthopus</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Eastern quoll (<em>Dasyurus viverrinus</em>)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>• Swamp wallaby (<em>Wallabia bicolor</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. gillettii</em> 14,15</td>
<td>Koala</td>
<td>NSW and Qld</td>
<td>Associated with lower PCV, lower body condition score, decreased survival in koalas with concurrent infections e.g., <em>Chlamydia</em> spp., KoRV 15</td>
</tr>
<tr>
<td></td>
<td>• Common brush-tailed possum (<em>Trichosurus vulpecula</em>)</td>
<td></td>
<td>Inflammation, tissue degeneration, necrosis in brush-tailed bettongs’ heart, skeletal muscle, oesophagus, tongue 17 impaired in brush-tailed bettong population decline 19</td>
</tr>
<tr>
<td></td>
<td>• Common wombat (<em>Vombatus ursinus</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Gilbert’s potoroo (<em>Potorous gilbertii</em>)</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>• Koala</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Quokka (<em>Setonix brachyurus</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Southern brown bandicoot (<em>Isoodon obesulus</em>)</td>
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</tr>
<tr>
<td></td>
<td>• Eastern quoll</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Spot-tailed quoll (<em>D. maculatus</em>)</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>• Brush-tailed bettong</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Brush-tailed rock-wallaby</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. copemani</em> 10-12,14-17,19,27</td>
<td>• Northern brown bandicoot (<em>I. macrourus</em>)</td>
<td>NSW, Qld, WA, ACT</td>
<td>Associated with significantly lower PCV in northern brown bandicoots 24</td>
</tr>
<tr>
<td></td>
<td>• Southern brown bandicoot</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Koala</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Tammar wallaby (<em>Macropus eugenii</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Western grey kangaroo (<em>M. fuliginosus</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Western quoll</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Brush-tailed bettong</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Gould’s wattled bat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Lesser long-eared bat</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. vegrandis</em> 16,17,20,22,24</td>
<td>• Northern brown bandicoot</td>
<td>NT, NSW, Qld, WA</td>
<td>Associated with significantly lower PCV in northern brown bandicoots 24</td>
</tr>
<tr>
<td></td>
<td>• Southern brown bandicoot</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Koala</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Tammar wallaby</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Western grey kangaroo</td>
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<tr>
<td></td>
<td>• Western quoll</td>
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<td></td>
<td>• Brush-tailed bettong</td>
<td></td>
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<tr>
<td></td>
<td>• Gould’s wattled bat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Lesser long-eared bat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td>Linked Animals</td>
<td>Distribution</td>
<td>Notes</td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
<td>--------------</td>
<td>-------</td>
</tr>
<tr>
<td><em>T. noyesi</em></td>
<td>Black flying-fox (<em>Pteropus alecto</em>)&lt;br&gt;Little red flying-fox (<em>P. scapulatus</em>)</td>
<td>NT, NSW, Qld, Vic., WA, ACT</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. thylacis</em></td>
<td>Northern brown bandicoot</td>
<td>Qld</td>
<td>Unknown</td>
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<tr>
<td><em>T. pteropi</em></td>
<td>Black flying-fox</td>
<td>Qld</td>
<td>Unknown</td>
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<td><em>T. hipposideri</em></td>
<td>Dusky leaf-nosed bat (<em>Hipposideros ater</em>)</td>
<td>Qld</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. teixeirae</em></td>
<td>Little red flying-fox</td>
<td>Qld</td>
<td>Anaemia, icterus, haemorrhage, acute haemoglobinuric nephrosis consistent with intravascular haemolysis reported in one individual</td>
</tr>
<tr>
<td>Trypanosoma sp. isolate ABF</td>
<td>Swamp wallaby</td>
<td>NSW</td>
<td>Unknown</td>
</tr>
<tr>
<td>Trypanosoma sp.</td>
<td>Eastern barred bandicoot (<em>Perameles gunnii</em>)</td>
<td>Tas., Vic.</td>
<td>Unknown</td>
</tr>
<tr>
<td>Trypanosoma sp.</td>
<td>Golden bandicoot (<em>I. auratus</em>)&lt;br&gt;Shark Bay mouse (<em>Pseudomys fieldi</em>)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>Trypanosoma sp.</td>
<td>Brush-tailed rock-wallaby</td>
<td>Vic.</td>
<td>Unknown</td>
</tr>
<tr>
<td>Trypanosoma sp.</td>
<td>Swamp wallaby</td>
<td>Vic.</td>
<td>Unknown</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
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<td>---------</td>
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<td>Unknown</td>
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<tr>
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<td>Trypanosoma sp.</td>
<td>Pygmy planigale (Planigale maculata)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>Trypanosoma sp.</td>
<td>Eastern bettongs, Eastern grey kangaroo</td>
<td>ACT</td>
<td>Unknown</td>
</tr>
<tr>
<td>Trypanosoma sp.</td>
<td>Water rat (Hydromys chrysogaster), Bush rat (Rattus fuscipes), Ash-grey mouse (Pseudomys albocinereus)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. lewisi</em></td>
<td>Short-beaked echidna (Tachyglossus aculeatus)</td>
<td>N/A</td>
<td>Acute trypanosomiasis similar to South American trypanosomiasis or Chaga’s disease (fever, anorexia, enlarged lymph glands); death due to heart failure.</td>
</tr>
<tr>
<td>T. evansi</td>
<td>Common brush-tailed possum</td>
<td>N/A</td>
<td>Death or sickness characterised by anorexia, ataxia, weakness, anaemia</td>
</tr>
</tbody>
</table>


*Species classification based on morphological characterisation only

^Exotic species introduced to Australia

# Animals experimentally infected with trypanosomes

N/A = Not available
1.1 Epidemiology

There is limited knowledge on the prevalence of trypanosomes in Australian mammals. Molecular studies have detected trypanosomes in the blood of up to 74% of koalas (*Phascolarctos cinereus*) and brush-tailed bettongs (*Bettongia penicillata*) (McInnes et al. 2011b; Botero et al. 2013; Barbosa et al. 2017b) and a recent survey conducted in the NT found prevalences of 27% and 24% in northern brown bandicoots (*Isoodon macrourus*) and common brush-tailed possums (*Trichosurus vulpecula*) respectively (Barbosa et al. 2017a). High prevalence has also been reported in platypuses (*Ornithorhynchus anatinus*) (86%) and various species of bats (82%) (Paparini et al. 2014; Austen et al. 2015b). Determining the true prevalence of trypanosomes in native mammals, especially rare species, is challenging given the relatively small sample sizes and restricted geographical distribution of most investigations conducted to date. Furthermore, prevalence data may vary between different studies, influenced by the techniques used for parasite detection and due to intermittent parasitaemia during the natural course of infection (Botero et al. 2013; Barbosa et al. 2017a).

However, studies to date have indicated that Australian trypanosomes appear to have a widespread geographic distribution, having been recorded in all states (Thompson et al. 2014a; Barbosa et al. 2017a; Barbosa et al. unpublished), except SA where limited investigations have been conducted. Additionally, there are many vertebrate populations across the Australian mainland and islands that have not yet been examined, meaning that current knowledge on the biogeography of Australian trypanosomes is far from complete.

Whilst there is no conclusive evidence on the identity of invertebrate vectors of trypanosomes in Australian native species, various ectoparasites including fleas, tabanid flies and several tick species (*Ixodes* spp., *Haemaphysalis* spp., *Amblyomma triguttatum*) have been reported as potential vectors (Mackerras 1959; Noyes et al. 1999; Hamilton et al. 2005; Austen et al. 2011; Paparini et al. 2014; Botero et al. 2016a; Barbosa et al. 2017b).

In terms of transmission dynamics, trypanosomes are divided in two groups: Salivaria and Stercoraria. The former undergo morphological and physiological transformation in the salivary glands of the vectors and are transmitted by inoculation during a blood meal; whereas the latter reside in the invertebrate vector’s gut and are released in the faeces and deposited on the skin of the host. The trypanosomes then penetrate the skin and disperse throughout the body. Another possible route of transmission is via ingestion of an infected vector by the host (Hoare 1972). The transmission dynamics of most Australian indigenous trypanosomes are unknown; however, the recent detection of *T. copemani* intact trypomastigotes in the faeces of *I. australiensis* after 30 d of incubation suggested that transmission is likely to be contaminative via tick faeces. (Austen et al. 2011).

1.2 Pathogenesis and clinical impacts

Whilst subclinical trypanosome infections are common in wildlife, disease can result in morbidity and mortality, particularly in immunocompromised hosts. Haemolytic anaemia is common and may be a consequence of physical effects of the parasites (e.g. mechanical injury to erythrocytes and release of proteases) or due to extravascular haemolysis arising from antibody-mediated erythrocyte destruction (Clark et al. 2004). In addition to the effects on erythrocytes, trypanosomes can invade
host cells (e.g. *T. copemani*), causing tissue inflammation, reducing fitness of the host and thereby potentially increasing susceptibility to predation (Botero *et al.* 2013).

Trypanosomes can also potentiate the effects of concurrent infections by compromising the immune system of their hosts (Khan and Lacey 1986; Goossens *et al.* 1997; Carrera *et al.* 2009). Trypanosome-induced immunosuppression involves various mechanisms of depression of cellular and humoral immune responses, including quantitative, biochemical and functional changes of T cells, B cells and macrophages (Zuniga *et al.* 2000; Vincendeau and Bouteille 2006).

Although most endemic trypanosomes identified to date are thought to be non-pathogenic (Thompson *et al.* 2014a; Cooper *et al.* 2016), there are several documented cases suggesting that some of these species have potential to be pathogenic. Additionally, exotic trypanosome species may also have a negative impact upon the health of naive mammalian hosts in Australia (Abbott 2006; Thompson *et al.* 2014a). *Trypanosoma lewisi* is the only exotic trypanosome identified in Australian native mammals to date, and has been associated with the extinction of endemic rodents.

### 1.2.1 *Trypanosoma lewisi* and extinction of endemic rats of Christmas Island

Following the unintentional introduction of black rats (*Rattus rattus*) infected with *T. lewisi* and their flea vectors to Christmas Is. in the early 1900s, two endemic rodent species, Maclear’s (*R. macleari*) and bulldog (*R. nativitatis*) rats became extinct. Reports at the time described Maclear’s rats being frequently found sick or moribund and heavily infected with trypanosomes and demonstrating pathological changes consistent with trypanosome infection at necropsy (Andrews 1909). A century after their extinction, molecular analysis of ancient DNA suggested that native trypanosomes were absent from the endemic rodents on Christmas Island prior to introduction of the black rat (Wyatt *et al.* 2008), lending weight to the hypothesis that *T. lewisi* infection contributed to the extinctions.

### 1.2.2 *Trypanosoma* spp. and koala morbidity

Although trypanosome infections in koalas may be asymptomatic, clinical signs of trypanosomiasis have been reported and include poor body condition and regenerative anaemia due to extravascular haemolysis. Trypanosomes are identified on blood films with a regenerative erythroid response evident in blood and bone marrow. Some cases progressed to developing neurological signs such as nystagmus, tremors and seizures; however, it is unclear if these signs could be attributed to the trypanosomes. Histopathology revealed small organisms in the liver and central nervous system suggestive of trypanosome intracellular amastigotes, however their identity could not be confirmed. Other findings included lymphocytic/plasmacytic choroiditis in the animals with neurological signs, with putative trypanosomes in some of the choroid vessels (A Gillett pers. comm.).

A recent study provided further evidence that trypanosome infections may impact koala health and survival and may be contributing to the decline of koala populations in eastern Australia (McInnes *et al.* 2011b). A significant association was reported between infection with *T. gilletti* and low PCV and body condition scores in koalas with signs of concurrent diseases (chlamydiosis, bone marrow dysplasia or KoRV-associated immune deficiency). This suggests that normally benign trypanosome infections may become pathogenic in koalas that are immunosuppressed or concurrently infected with other pathogens, particularly KoRV. Alternatively, the trypanosome infection may be inducing immunosuppression and therefore potentiating the effects of concomitant diseases (McInnes *et al.* 2011b).
Mixed infections with up to six *Trypanosoma* spp., including *T. irwini*, *T. gilletti*, *T. copemani*, *T. vegrandis* and *T. noyesi* have been reported in koalas (McInnes et al. 2011a, McInnes et al. 2011b, Barbosa et al. 2016b, Barbosa et al. 2017b). Further research is required to unravel the biological interactions involved in mixed trypanosome infections and in mixed chlamydial/KoRV and trypanosome infections.

### 1.2.3 *Trypanosoma* infection and the brush-tailed bettong population decline

The brush-tailed bettong (woylie) is critically endangered and has undergone a rapid population decline of 90% (Thompson et al. 2014b). It has been suggested that trypanosomes may have played a role in this decline. Recent studies provide evidence that *T. copemani* amastigote stages can migrate to and replicate in a range of brush-tailed bettong tissues including heart, skeletal muscle, oesophagus and tongue, resulting in inflammation and tissue degeneration (Botero et al. 2013; Botero et al. 2016b). These effects may reduce fitness and coordination, making them more susceptible to predation (Botero et al. 2013). This observation has been further supported by a temporal association between *T. copemani* prevalence and declines of the Kingston indigenous brush-tailed bettong population in the Upper Warren region in WA (Thompson et al. 2014b).

A recent study revealed a relationship between *T. copemani* infection and the functional efficiency of innate immunity (phagocytosis) in brush-tailed bettongs. *T. copemani* infected bettongs had higher faecal cortisol metabolites (FCM) associated with a lower phagocytosis index. However, when the bettongs were trypanosome negative, higher FCM was associated with higher phagocytosis index. This suggests that during periods of *Trypanosoma* parasitaemia, the animals are more vulnerable to the immunosuppressive effects of glucocorticoids. Alternatively, a combination of host stress physiology and infection status may affect the efficiency of leukocyte function (Hing et al. 2016).

Pathological changes in brush-tailed bettongs may also occur with mixed infections involving *T. vegrandis* and *T. noyesi*, suggesting a greater immunosuppressive effect or enhanced pathogenicity with mixed infections (Botero et al. 2013). The finding of a higher prevalence of mixed infections involving *T. copemani*, *T. vegrandis* and *T. noyesi* in a declining brush-tailed bettong population compared to one with stability seems to support this theory (Botero et al. 2013). Conversely, it has been hypothesised that interspecific competition may exist between *T. copemani* and *T. vegrandis*, where an existing *T. vegrandis* infection may moderate the sequential establishment of *T. copemani* (Thompson et al. 2014b). As with koalas, the clinical impact of trypanosome co-infections on brush-tailed bettong health warrants further investigation.

### 1.2.4 Trypanosomiasis in a little red flying fox

The first case report of a trypanosome infection associated with clinical disease in bats involved an adult female little red flying fox (*Pteropus scapulatus*), found on the ground in Redcliffe, Qld. The animal presented with icterus and severe anaemia. Necropsy and histological findings were consistent with trypanosome infection of lymphoid tissue and intravascular haemolysis (Mackie et al. 2017). Examination of a blood smear by light microscopy revealed the presence of numerous trypanosomes and molecular analysis revealed the parasite was a novel species (Mackie et al. 2017). More extensive phylogenetic analysis confirmed the species status of this novel trypanosome, and
was named *T. teixeirae*. It was shown to be closely related to the pathogenic *T. cruzi* of South America (Barbosa et al. 2016a).

### 1.2.5 *Trypanosoma vegrandis* and anaemia in northern brown bandicoots

A recent investigation of haemoprotezoan parasites in native mammals from northern Australia demonstrated that northern brown bandicoots positive for *T. vegrandis* were more likely to have lower PCVs than negative individuals. No other clinical signs were associated with infection (Barbosa et al. 2017a).

### 1.2.6 *Trypanosoma* sp. and morbidity in eastern bettongs and mortality of eastern grey kangaroos

During an investigation of a recent mortality event of eastern grey kangaroos (*Macropus giganteus*) and weight loss in eastern bettongs (*B. gaimardi*) in the ACT, a novel *Trypanosoma* sp. was detected in 5/10 kangaroos and 6/9 bettongs. The novel species was genetically distinct from, but most closely related to *T. copemani* (genetic distance = 14%) (Barbosa et al. unpublished). Haematology revealed regenerative anaemia suggestive of chronic blood loss. The role of *Trypanosoma* sp. in the mortality and morbidity events is not clear and its potential involvement should be considered with caution, in the context of a broader investigation into these events. For instance, infections with *Theileria* sp. (see section 2.2.6) and moderate endoparasitism were also observed, as well as unusual climatic conditions (i.e. a prolonged period of sub-zero temperatures) and low pasture biomass during the winter period (Barbosa et al. unpublished; T Portas pers. comm.).

### 1.3 Diagnosis

Giemsa or Wright’s stained blood smears are used for detection of trypanosomes by light microscopy (Fig. 24.1a and 24.2). Although convenient and readily available, microscopic detection of trypanosomes is relatively insensitive particularly in chronic stages of infection and in cases of low level parasitaemia (e.g. *T. noyesi*) (Botero et al. 2016a). Various molecular techniques are now available for detection of trypanosomes. For research purposes, electron microscopy is particularly useful for ultrastructural characterisation of the parasites (Fig. 24.1b).

PCR assays are able to amplify very small amounts of trypanosome DNA in blood. Additionally, PCR combined with Sanger sequencing can be used for species identification. Morphological characterisation is insufficient for species identification, due to overlapping morphometry between species and polymorphic life cycle stages in vertebrate hosts (Thomson et al. 2013; Austen et al. 2015a). PCR and sequence analysis of partial fragments of the 18S rRNA gene are the most widely used diagnostic method for *Trypanosoma* identification. However, amplification at additional loci, such as the nuclear glyceraldehyde 3-phosphate dehydrogenase gene and the mitochondrial cytochrome B gene, can be adopted for a more robust genetic characterisation (Hamilton et al. 2004; Botero et al. 2016a; Cooper et al. 2016).

Although Sanger sequencing of PCR amplicons, generated by universal primers for the genus *Trypanosoma*, is a commonly used method for species identification, it does not allow
characterisation of multiple genotypes involved in mixed trypanosome infections, which are common in brush-tailed bettongs and koalas (McInnes et al. 2011a; McInnes et al. 2011b; Paparini et al. 2011; Botero et al. 2013; Barbosa et al. 2017b). In these cases, application of alternative methods such species-specific PCR, cloning or next-generation sequencing are necessary to characterise mixed infections (McInnes et al. 2011b; Paparini et al. 2011; Barbosa et al. 2017b).

1.4 Treatment and control

There are no specific treatment recommendations for trypanosomiasis in Australian mammals. Supportive care; including blood transfusions, IV fluid therapy, nutritional and thermal support are useful. Additional symptomatic treatment, such as the use of diazepam in koalas exhibiting neurological signs, is recommended (A Gillett pers. comm.).

Prevention and control of trypanosome transmission through limiting exposure to invertebrate vectors is generally not feasible. However, monitoring and surveillance to gather baseline data on prevalence and genetic diversity of trypanosomes in Australian mammal populations and their vectors is crucial for conservation management, particularly in translocated populations.

1.5 Zoonotic potential

There is currently no evidence that Australian native trypanosomes are zoonotic. A recent study however, has shown that T. copemani is naturally resistant to human serum and therefore may be potentially zoonotic (Austen et al. 2015c).

1.6 Biosecurity concerns

The potential negative impact of exotic trypanosomes such as T. lewisi, T. cruzi and T. evansi, if they established and spread within Australian native mammals presents a biosecurity concern (Thompson 2013; Thompson et al. 2014). T. lewisi is the only exotic species already introduced and found in native mammals (Thompson et al. 2014).

In 1907, surra-infected camels were imported into WA; fortunately, this infection was diagnosed quickly and T. evansi was eradicated prior to establishment (Mackerras 1959). If T. evansi had established in Australia, the consequences for Australian native mammals and domestic livestock could have been devastating (O’Donoghue and Adlard 2000; Reid et al. 2001; Reid 2002).

The zoonotic pathogen T. cruzi is genetically very similar to some Australian trypanosomes, particularly T. noyesi and T. teixeirae. It has been hypothesised, for example, that the vector of T. noyesi could potentially transmit T. cruzi from humans (infected immigrants and travellers) to indigenous mammals (Thompson et al. 2014; Thompson and Thompson 2015). Experimental T. cruzi infection has been demonstrated in the short-beaked echidna (Tachyglossus aculeatus) and brush-tailed possum (Backhouse and Bolliger 1951), confirming the potential for disease and mortality of native mammals and the potential for native mammals to act as a reservoir for human infection (Thompson and Thompson 2015).

Continued surveillance and identification of vectors of Australian trypanosomes are required to ascertain the biosecurity risks of exotic trypanosomes establishing in Australia (Thompson and Thompson 2015).
2. Piroplasms

Piroplasms are tick-borne protozoan parasites that infect mammalian and avian erythrocytes. These organisms can cause significant economic losses due to high pathogenicity in domestic production animals and horses worldwide and severe clinical disease in companion animals (Colwell et al. 2011). Although the clinical significance of piroplasms in Australian native mammals is still relatively unknown, sporadic case reports have suggested that they can be pathogenic (Backhouse and Bolliger 1957; Barker et al. 1978; Dawood et al. 2013; Kessell et al. 2014; Donahoe et al. 2015).

The Order Piroplasmida includes three genera: Babesia, Theileria and Cytauxzoon. Of these, Babesia and Theileria are known to infect Australian mammals. Currently, there are 10 formally named species of piroplasms recorded in Australian mammals and genetic data from five un-named marsupial-derived piroplasms. The species’ names, known vertebrate hosts, geographic distribution and overall clinical significance are presented in Table 24.2.

Table 24.2: Piroplasms (Babesia and Theileria) of Australian native mammals
<table>
<thead>
<tr>
<th>Piroplasm species</th>
<th>Host(s)</th>
<th>Source/distribution</th>
<th>Clinical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Babesia tachyglossi</em> /</td>
<td>Short-beaked echidna (<em>Tachyglossus aculeatus</em>)</td>
<td>NSW, Qld, WA</td>
<td>Associated with a mortality event involving 12 captive individuals in NSW</td>
</tr>
<tr>
<td><em>Theileria tachyglossi</em></td>
<td>T. ornithorhynchi 3,5,6,8,18,20,21</td>
<td>NSW, Qld, Tas.</td>
<td>High parasitaemia associated with dehydration, anorexia and fatal haemolytic anaemia</td>
</tr>
<tr>
<td><em>B. macropus</em> 12,17,19,23</td>
<td>Platypus (<em>Ornithorhynchus anatinus</em>)</td>
<td>Qld, ACT</td>
<td>Severe clinical disease</td>
</tr>
<tr>
<td><em>B. thylacis</em> *</td>
<td>Agile wallaby (<em>Macropus agilis</em>)</td>
<td>Qld, WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>B. vogeli</em> 10,14</td>
<td>Dingo (<em>Canis lupus dingo</em>)</td>
<td>NT, WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Babesia sp.</em> 4,9,10</td>
<td>Agile antechinus (<em>Antechinus agilis</em>)</td>
<td>Vic.</td>
<td>Severe clinical disease</td>
</tr>
<tr>
<td><em>Babesia sp.</em> 15</td>
<td>Brush-tailed bettong (<em>B. penicillata</em>)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Babesia sp.</em> 22</td>
<td>Northern brown bandicoot</td>
<td>NT</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>T. brachyuri</em> 11</td>
<td>Quokka (<em>Setonix brachyurus</em>)</td>
<td>WA</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Theileria sp. isolate K1</em></td>
<td>Burrowing bettong (<em>B. lesueur</em>)</td>
<td>ACT, WA</td>
<td>Unknown</td>
</tr>
<tr>
<td>Species</td>
<td>Common Name</td>
<td>Location</td>
<td>Notes</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------------------------------------</td>
<td>----------</td>
<td>----------------------------</td>
</tr>
<tr>
<td><em>T. fuliginosa</em></td>
<td>Swamp wallaby</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. gilberti</em></td>
<td>Gilbert’s potoroo (<em>Potorous gilbertii</em>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. penicillata</em></td>
<td>Brush-tailed bettong</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>T. peramelis</em></td>
<td>Long-nosed bandicoot (<em>Perameles nasuta</em>)</td>
<td>Qld</td>
<td>Anaemia without apparent clinical illness</td>
</tr>
<tr>
<td><em>Theileria sp.</em></td>
<td>Long-nosed potoroo (<em>P. tridactylus</em>)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


*Species classification based on morphological characterisation only.
2.1 Epidemiology

The true prevalence of piroplasms in Australian native mammals is unknown although there have been recent studies of limited populations in geographically disparate regions. High prevalence of *T. gilberti* in free-ranging populations of the Gilbert’s potoroo (*Potorous gilbertii*) (100%, 16/16) and *T. penicillata* in brush-tailed bettongs (80.4%, 123/153) from WA (Lee *et al.* 2009; Rong *et al.* 2012) have been reported. In another study conducted on captive and free-ranging marsupial populations in WA, the overall detection rate of piroplasm-infected animals was 7.1% (8/113). Of these, a novel *Babesia* was identified in seven free-ranging brush-tailed bettongs and a single *Theileria* positive sample was identified from one of three captive burrowing bettongs (*B. lesueur*) (Paparini *et al.* 2012). Another study focusing on the health of free-ranging eastern bettongs (*B. gaimardi*) captured in Tas. and translocated to the ACT, examined blood films by microscopy and intraerythrocytic piroplasms were observed in 6.7% (4/60) of the animals (Portas *et al.* 2014). More recently, a molecular survey conducted on free-ranging mammals from the NT, identified a *Babesia* sp. in northern brown bandicoots, at a prevalence of 9.7% (Barbosa *et al.* 2017a).

Piroplasms in native mammals have been recorded in most states and territories of Australia (Table 24.2). Ticks are believed to be the main vectors, as they are ubiquitous and parasitise all vertebrate hosts of *Babesia* and *Theileria* species recorded to date. Ticks collected from infected animals have also been positive for piroplasms using molecular methods. In this context, native *Haemaphysalis* spp. and *Ixodes* spp. seem likely vector candidates (Dawood *et al.* 2013). Under natural conditions, piroplasms are transmitted from the invertebrate vector to the definitive host via the ticks’ saliva during feeding.

Small *Babesia* spp. found in Australian ticks exhibit transovarial transmission, in which the parasites migrate to the ovaries and are transmitted vertically from the gravid female to larvae. In contrast, *Theileria* spp. undergo only transstadial transmission, in which the parasite is passed on from one life stage (‘stadium’) to the next. Furthermore, while *Babesia* spp. only multiply in red blood cells, *Theileria* spp. undergo extra-erythrocytic schizogony in lymphocytes or macrophages, prior to invading erythrocytes (Uilenberg 2006; Sivakumar *et al.* 2014).

2.2 Pathogenesis and clinical significance

Although most piroplasms reported in Australian mammals appear non-pathogenic, there are some examples of morbidity and mortality associated with infection. Haemolytic anaemia and thrombocytopenia are recognised as the most typical pathology of piroplasmosis. These maybe the result of the physical effects of parasites on cells or the activation of an immune response which leads to cell destruction by macrophages (Clark *et al.* 2004). Clinical signs of piroplasmosis may include lethargy, reduced mentation, diarrhoea and neurological signs and may vary with species of parasite and host.

Interestingly, the pathogenesis of *B. macropus* in macropods appears to have similarities with that of *B. bovis* in cattle, characterised by sequestering of infected erythrocytes in the capillaries of organs, leading to low peripheral parasitaemia, disseminated intravascular coagulation and severe cerebral babesiosis (Dawood *et al.* 2013).
2.2.1 Babesia/Theileria tachyglossi and short-beaked echidna mortality

Piroplasms (B. tachyglossi and T. tachyglossi) found in short-beaked echidnas were originally described based solely on microscopy (Priestley 1915; Backhouse and Bolliger 1957, 1959; Mackerras 1959). At the time of the original reports, the genera Babesia and Theileria had not yet been defined, in regard to the existence of schizonts and/or transstadial vs. transovarial transmission. Molecular data suggests that only a single piroplasm species, believed to be T. tachyglossi, infects echidnas (Slapeta et al. 2017).

Infection with piroplasms was implicated in the deaths of 12 captive short-beaked echidnas (Backhouse and Bolliger 1957). Subsequently, there have been no further reports of pathological effects of piroplasms in echidnas despite these parasites being frequently observed in blood films (L Vogelnest pers. comm.). Monotreme populations in Australia remain relatively understudied with respect to prevalence and clinical impact of piroplasm infection.

2.2.2 Anaemia in bandicoots associated with Theileria peramelis

Although natural infections with T. peramelis have been recorded in southern brown (I. obesulus) and long-nosed bandicoots (Perameles nasuta), there is only one report associating experimental infection with T. peramelis and anaemia in these species. The anaemia observed was strongly regenerative, characterised by increased anisocytosis and immature erythrocytes (Mackerras 1959).

2.2.3 Babesia infection and fatal anaemia in antechinuses

Infection with a Babesia sp. has been associated with clinical piroplasmosis in the brown antechinus (Antechinus stuartii) and male agile antechinuses (A. agilis) undergoing physiological stress in the post mating period. These animals presented with decreased PCV, haemoglobinuria and haemosiderosis of the lung and spleen (Cheal et al. 1976, Barker et al. 1978).

2.2.4 Theileria ornithorhynchi and fatal anaemia in platypuses

Although T. ornithorhynchi is generally considered non-pathogenic, this parasite has been associated with fatal haemolytic anaemia in an orphaned juvenile platypus. The animal had concurrent fungal dermatitis and a heavy tick burden (Kessell et al. 2014). Association of T. ornithorhynchi with disease is further supported by a recent report of high (10–15%) parasitaemia in three diseased male wild platypuses (two juvenile and one sub-adult). One of them was also infected with Eimeria sp. The animals were dehydrated and anorexic. Histopathology revealed severe suppurative enteritis and active spleen with increased erythropagocytosis (Slapeta et al. 2017).

2.2.5 Babesia macropus and severe babesiosis in macropods

Although subclinical infection with Babesia spp. appears to be relatively common in macropods, B. macropus infection has been associated with a syndrome of haemolytic anaemia and debility in hand-reared and free-ranging juvenile eastern grey kangaroos, agile wallabies (M. agilis), red-necked wallabies (M. rufogriseus) and swamp wallabies (Wallabia bicolor). Clinical signs included severe pallor, lethargy, polydipsia, polyuria, neurological signs and death (Vogelnest and Portas 2008; Dawood et al. 2013; Donahoe et al. 2015). PCV of less than 10% are commonly observed in affected
animals, which may also be hypoproteinaemic with total protein levels as low as 30 g/L (Vogelnest and Portas 2008).

Other variable, clinical pathological findings associated with this disease included thrombocytopenia, neutropenia, hyperamylasemia, azotaemia and bilirubinaemia. The neurological signs observed in some kangaroos may be related to intravascular sequestration of parasitised erythrocytes within the central nervous system. Potential pathogenic mechanisms include hypoxia, inflammatory cytokine release following obstructive sequestration of erythrocytes and endothelial damage (Donahoe et al. 2015). Necropsy findings included diffuse pallor of the carcass and visceral organs, thin watery blood, widespread petechiae, ecchymoses, tissue oedema, splenomegaly and generalised lymphadenomegaly (Donahoe et al. 2015).

As most of the infected kangaroos were captive/hand-reared, it is likely that the stress of handling, transportation and captivity contributed to the development of disease. Furthermore, kangaroos hand-reared by carers lack the relevant maternal antibodies and also have less exposure to naturally occurring parasites compared to free-ranging kangaroos, therefore rendering them more susceptible to infection (Donahoe et al. 2015). Alternatively, it is possible that the disease was more likely to be detected in captive animals than from free-ranging animals.

2.2.6 *Theileria* sp. and morbidity in eastern bettongs and mortality of eastern grey kangaroos

A novel *Theileria* sp. has been detected in 4/9 eastern bettongs and 4/10 eastern grey kangaroos involved in recent morbidity and mortality events, respectively, in the ACT (Barbosa et al. unpublished). Amongst other clinical signs, the animals exhibited regenerative anaemia which may be associated with *Trypanosoma* (see section 1.2.6) and *Theileria* infections. However, the role of these parasites in the morbidity and mortality events is unclear due to the presence of concurrent infections and potential environmental causes such as unusual climatic conditions and lower pasture biomass (T Portas pers. comm.).

2.3 Diagnosis

Light microscopy is the primary method used for detection of piroplasms, however it lacks sensitivity, particularly in chronic or subclinical cases (Clark et al. 2004). Morphological features within intra-erythrocytic piroplasms are also indistinguishable among different genera and species (Homer et al. 2000). Schizonts of *Theileria* spp., present in leukocytes, may also be very difficult to find. Additionally, piroplasm morphology in Australian mammals is highly pleomorphic, with intra-erythrocytic organisms characterised as ring-shaped trophozoites and/or pairs or tetrads of pyriform shaped merozoites (Fig. 24.3a). Even though the infection typically affects less than 1% of erythrocytes (Clark et al. 2004), piroplasms may exhibit high parasitaemia, as observed for *T. gilberti* in the Gilbert’s Potoroo (Lee et al. 2009) (Figure 24.3b).
Molecular and phylogenetic analyses are required for confirmation of genus and species (Schnittger et al. 2003). Molecular methods are significantly more sensitive than microscopy (Clark et al. 2004). PCR assays targeting the 18S rRNA gene are the most widely used molecular method for the detection and characterisation of piroplasms in Australian mammals (Paparini et al. 2012; Dawood et al. 2013). In addition, a next-generation sequencing assay was recently developed to study the diversity of apicomplexan parasites (including piroplasms) in platypuses and echidnas (Slapeta et al. 2017).

2.4 Treatment and control

While a wide range of antiprotozoal drugs has been used for the treatment of piroplasmosis in domestic animals, there is currently no robust scientific evidence of effectiveness of these drugs in Australian mammals, or awareness of potential toxic effects. There are occasional reports of parenteral treatment with imidocarb dipropionate; however, response to this treatment remains anecdotal and unevaluated (Vogelnest and Portas 2008; Dawood et al. 2013; Kessell et al. 2014; Donahoe et al. 2015).

Suggested treatment regimens include provision of supportive care, including blood transfusions, intravenous fluids, improved nutrition and warmth if the animal is hypothermic (Vogelnest and Portas 2008).

As with trypanosomes, prevention of piroplasm transmission by vector control is not generally feasible. Administration of prophylactic doses of anti-protozoal medication has been suggested for macropods before and after translocation to prevent development of clinical babesiosis (Donahoe et al. 2015). Awareness of the prevalence and genetic diversity of piroplasms is crucial for appropriate conservation management and translocation strategies.
2.5 Zoonotic potential

The first autochthonous case of human babesiosis reported in Australia was diagnosed in 2010, and the aetiological agent was identified as *B. microti* (Senanayake et al. 2012). Even though several *Babesia* spp. have been isolated from Australian mammals, there is currently no reservoir identified for this zoonotic *Babesia* in Australia. There is no scientific evidence that *Theileria* spp. infecting Australian mammals are zoonotic.

Acknowledgements

We thank Dr Andrea Paparini, Dr Jill Austen and Dr Amber Gillett for their valuable contributions to this chapter.

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https://doi.org/10.1016/j.parint.2016.03.004
Increased genetic diversity and prevalence of co-infection with *Trypanosoma* spp. in koalas (*Phascolarctos cinereus*) and their ticks identified using next-generation sequencing (NGS)

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Abstract

Infections with *Trypanosoma* spp. have been associated with poor health and decreased survival of koalas (*Phascolarctos cinereus*), particularly in the presence of concurrent pathogens such as *Chlamydia* and koala retrovirus. The present study describes the application of a next-generation sequencing (NGS)-based assay to characterise the prevalence and genetic diversity of trypanosome communities in koalas and two native species of ticks (*Ixodes holocyclus* and *I. tasmani*) removed from koala hosts. Among 168 koalas tested, 32.2% (95% CI: 25.2–39.8%) were positive for at least one *Trypanosoma* sp. Previously described *Trypanosoma* spp. from koalas were identified, including *T. irwini* (32.1%, 95% CI: 25.2–39.8%), *T. gilletti* (25%, 95% CI: 18.7–32.3%), *T. copemani* (27.4%, 95% CI: 20.8–34.8%) and *T. ve grandi s* (10.1%, 95% CI: 6.0–15.7%). *Trypanosoma noyesi* was detected for the first time in koalas, although at a low prevalence (0.6% 95% CI: 0–3.3%), and a novel species (*Trypanosoma* sp. AB-2017) was identified at a prevalence of 4.8% (95% CI: 2.1–9.2%). Mixed infections with up to five species were present in 27.4% (95% CI: 21–35%) of the koalas, which was significantly higher than the prevalence of single infections 4.8% (95% CI: 2–9%). Overall, a considerably higher proportion (79.7%) of the *Trypanosoma* sequences isolated from koala blood samples were identified as *T. irwini*, suggesting this is the dominant species. Co-infections involving *T. gilletti, T. irwini, T. copemani, T. vegrandis* and *Trypanosoma* sp. AB-2017 were also detected in ticks, with *T. gilletti* and *T. copemani* being the dominant species within the invertebrate hosts. Direct Sanger sequencing of *Trypanosoma* 18S rRNA gene amplicons was also performed and results revealed that this method was only able to identify the genotypes with greater amount of reads (according to NGS) within koala samples, which highlights the advantages of NGS in detecting mixed infections. The present study provides new insights on the natural genetic diversity of *Trypanosoma* communities infecting koalas and constitutes a benchmark for future clinical and
epidemiological studies required to quantify the contribution of trypanosome infections on koala survival rates.

Introduction

Trypanosomes are blood-borne protozoans of veterinary and medical clinical significance. In Australia, special attention has recently been given to their potential impact on health of native wildlife [1–8]. The koala (Phascolarctos cinereus) is an iconic Australian marsupial that is under threat of extinction across two thirds of its range, with population declines of over 50% being reported in some states [9]. Koala populations in New South Wales (NSW) and Queensland (Qld) have been listed as vulnerable under the NSW Threatened Species Conservation Act 1995 and the Qld Nature Conservation (Wildlife) Regulation 2006, respectively [10, 11]. Chlamydiosis caused by Chlamydia pecorum is the main disease contributing to koala population decline [12], acting in synergy with many other variables adversely affecting koala survival (such as concurrent infections with koala retrovirus-KoRV, habitat loss, vehicle collisions, climate change and dog attacks) [13, 14]. Preliminary data suggests that trypanosome infections may also be compromising the health of wild koalas, particularly those with clinical signs of concurrent diseases, thereby also contributing to population decline events [3].

Four Trypanosoma spp. in either single or mixed infections have been recorded in koalas to date: Trypanosoma irwini, T. gilletti, T. copemani and T. vegrandis, at prevalences ranging from 4.4% to 71.1% [3, 15–17]. The most common molecular method for diagnosing trypanosome infection is direct Sanger sequencing of PCR amplicons obtained using Trypanosoma generic primers. This method, although fast and sensitive, can impede the detection of multiple distinct co-amplified genetic variants, meaning that trypanosome co-infections can remain undetected, particularly when one trypanosome genotype is present at a lower abundance than the other [18–20]. Thus, previous studies have relied on alternative techniques such as culturing, cloning or species-specific PCR assays to resolve co-infections with trypanosomes [3, 4, 17, 21]. Such methods can, however, be relatively costly and time-consuming when screening a large amount of samples and species-specific PCR assays are limited to known species, which greatly limits the possibility of uncovering novel or rare species. On the other hand, next-generation sequencing (NGS) allows high-throughput parallelization of sequencing reactions and is therefore helpful to accurately determine the prevalence and genetic diversity of Trypanosoma spp. in wildlife communities and potential vectors.

Although NGS is a well-established method for profiling bacterial communities, with the exception of Plasmodium in mosquitoes, relatively few studies have applied this technology in the diagnostics of protozoal infections [22–25]. To the best of the authors’ knowledge, no previous published studies have used an NGS approach to investigate mixed trypanosome infections in wildlife, particularly koalas, or in potential vectors such as ticks. The development of such protocol is therefore critical, not only from a conservation awareness perspective, but also in a One Health context, as NGS has also the potential to uncover zoonotic pathogens that may be present in relatively low abundance within wildlife and tick samples.

The present study aimed to design and validate a novel NGS-based assay targeting the 18S rRNA locus to audit Trypanosoma communities in koala blood samples and ticks removed from koala hosts. This NGS assay was then used to determine the prevalence and genetic diversity of Trypanosoma sp. in koalas from eastern Australia and identify potential vectors for Trypanosoma.
Materials and methods

Sampling

A total of 168 blood samples and 91 ticks collected from koalas were investigated during the present study. Blood samples were collected during routine clinical procedures from 161 koalas (84 females and 77 males) admitted to the Australia Zoo Wildlife Hospital (AZWH), Beerwah, Qld between December 2010 and December 2011. An additional seven blood samples were collected from koalas (three females and four males) that presented to the Koala Hospital, Port Macquarie, NSW, between October 2014 and February 2015. Most of these animals originated from south-east Qld or northern NSW. For blood collection, the koalas were induced for anaesthesia with an intramuscular administration of alfaxalone (Alfaxan1 CD RTU, Jurox Australia) at a dose rate of 3 mg/kg. Anaesthesia was maintained using a combination of 1.5% isofluorane and oxygen at 1.5 L/min delivered by either mask or endotracheal intubation. Approximately 0.5–1 ml of blood was collected by venepuncture of the cephalic vein.

Of the 91 ticks collected from koalas between December 2009 to August 2014; 81 were collected at the AWZH and the remaining 10 were supplied by Endeavour Veterinary Ecology Pty Ltd, Toorbul, Qld. The ticks were stored in 70% ethanol after collection until DNA extraction. Due to differences in sampling sites, collection dates, and protocols (i.e. ticks were collected opportunistically and blood was collected during routine veterinary procedures that required anaesthesia), the ticks and blood from corresponding hosts (i.e. koala parasitized by that particular tick) were collected concurrently in only 15 cases. All aspects of sample collection were approved by the Murdoch University Animal Ethics Committee (permit numbers W2284/09 and W2636/14).

Tick identification

Tick instars and species were microscopically examined with an Olympus SZ61 stereomicroscope (Olympus, Center Valley, PA, USA), with a Schott KL 1500 LED light source (Schott AG, Mainz, Germany), and morphologically identified using standard taxonomic keys [26, 27]. All ticks analysed in the present study belonged to the family Ixodidae; of which 56 (61.5%) were identified as *Ixodes tasmani* and 35 (38.5%) as *I. holocyclus*. In total, 82 (93.8%) of ticks were females. Of these, 49 (59.8%) belonged to the species *I. tasmani* and 33 (40.2%) to the species *I. holocyclus*. Only 9 ticks (7 *I. tasmani* and 2 *I. holocyclus*), were males. No nymphs or larvae were observed.

DNA extraction

Genomic DNA was isolated from 200 μl of whole blood from koalas using a MasterPure™ DNA Purification Kit (EPICENTRE Biotechnologies, Madison, Wisconsin, U.S.A.), according to the manufacturer’s instructions. DNA was eluted in 35 μl of TE buffer and stored at -20˚C until use.

Prior to DNA isolation, ticks were surface sterilised in 10% sodium hypochlorite, washed in 80% ethanol and DNA-free PBS, and air-dried. Individual ticks were then cut into quarters with a sterile scalpel, placed in a 2 ml microtube with a 5 mm steel bead, snap frozen in liquid nitrogen for 1 min and homogenized by shaking at 40 Hz for 1 min. Genomic DNA was isolated from tick homogenates using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations. Extraction reagent blank controls were included alongside all DNA extractions. DNA extraction, PCR setup, and DNA handling procedures were all performed in separate physically contained exclusion hoods, and post-PCR procedures were performed in a separate dedicated laboratory.
Trypanosoma 18S rRNA gene metabarcoding

Partial Trypanosoma 18S rRNA (18S) gene sequences (~350 bp) were PCR amplified from koala and tick DNA samples using a hemi-nested PCR assay utilizing the primary primers S825F [28] and TryAll R1 (5′-GACTGTAACCTCAAAGCTTTTCGC-3′) (designed during the present study), and hemi-nested primers S825F and S662R [28]. Hemi-nested PCR primers also contained Illumina MiSeq adapter sequences on the 5' end, as per standard protocols for the MiSeq platform (Illumina Demonstrated Protocols: Metagenomic Sequencing Library Preparation). PCRs were performed in 25 μl volumes containing PCR buffer, 1.5 mM MgCl₂, 1 mM dNTPs, 0.8 μM of each primer, 0.04 U/ml Taq DNA polymerase (Fisher Biotec, Australia) and 2 μl of DNA (primary PCR).

Primary PCR products were electrophoresed through a 2% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA), visualized with a dark reader trans-illuminator (Clare Chemical Research, USA), and products corresponding to the correct length were excised, purified using a QIAquick gel extraction kit (QIAGEN, Germany). Hemi-nested PCRs used 1 μl of purified primary product as a template. Primary and hemi-nested PCRs were performed with an initial denaturation at 95˚C for 3 min followed by 40 cycles of denaturation at 95˚C for 30 s, annealing at 53˚C (primary PCR) or 55˚C (hemi-nested PCR) for 30 s, and extension at 72˚C for 30 s, followed by a final extension of 72˚C for 5 min. Hemi-nested PCR products were electrophoresed and purified as for the primary PCR products.

DNA samples from seven koalas (previously obtained from the Australia Zoo Wildlife Hospital) and known to harbour dual or triple trypanosome infections with T. irwini, T. gilletti and T. copemani (as determined by Sanger sequencing of cloned PCR products and/or species-specific PCR’s) [3, 16], were included in this experiment as positive controls. In addition, DNA from the pathogenic T. cruzi was included as a representative of a distantly related species from those known to infect koalas, to make sure primers were able to pick up a wide range of species including those within this important clade of trypanosomes. No-template and extraction blank controls were included in all PCR assays.

Resulting Trypanosoma 18S amplicons from each sample were then uniquely indexed with DNA barcodes and prepared for sequencing according to Illumina recommended protocols (Illumina Demonstrated Protocol: Metagenomic Sequencing Library Preparation), and sequenced on an Illumina MiSeq using 500-cycle V2 chemistry (250 bp paired-end reads), following the manufacturer’s recommendations.

Bioinformatics analysis

Paired-end reads were overlapped (50 bp minimum overlap length, no mismatches allowed) and merged in USEARCH v8.0.1623 [29]. Sequences were then imported into Geneious 8.0.4 [30], where S825F and S662R primer sequences and distal bases were trimmed from the 5’ and 3’ ends of the reads. Only sequences containing the correct primer sequences (no SNPs) were retained for further analyses. In USEARCH v8.0.1623, the sequences were quality filtered (sequences with >2% expected errors were excluded from the dataset), de-multiplexed and singletons were removed on a per-run basis.

Trypanosoma operational taxonomic units (OTUs) were generated by clustering sequences with ≥ 99% similarity, using the UPARSE algorithm [31]. This threshold was determined based on the smallest genetic divergence at the 18S locus studied between trypanosome species found in koalas to date, according to an in-house database comprising curated Trypanosoma 18S sequences retrieved from GenBank. A list of species, isolate names and respective GenBank accession codes are shown in S1 Table.
After chimera filtering was carried out, the retained OTUs were BLAST-searched against the same in-house trypanosome library. Taxonomy was assigned to a species level in QIIME v.1.9.1 [32], using the BLAST algorithm (e = 0.0001). The sequences not assigned to a species level were classified as either “Trypanosoma sp.” (i.e. assigned to a genus-level only) or “no BLAST hits” (i.e. unassigned), and further inspected during the phylogenetic analysis.

In order to design an effective taxonomic assignment method for the present study, several algorithms and stringency parameters available in QIIME v.1.9.1 were tested, starting from highly stringent protocols (e.g. BLAST with 100% similarity threshold; BLAST with 99% similarity threshold associated with a 2/3 consensus fraction using the UCLUST algorithm). To verify the sensitivity and specificity of the algorithm selected upstream, fast phylogenetic trees were drawn in Geneious 8.0.4 (function: Fast Tree), using all assigned and unassigned OTU sequences, after each batch of test results was generated (data not shown). A number of incongruences between the NGS-BLAST taxonomic assignment and taxonomy revealed by phylogenetic analysis were observed when using more stringent parameters. For instance, many sequences classified as “unassigned” due to a lower genetic identity compared to a determined reference species (<98%), could be assigned to species according to the phylogenetic reconstructions. Therefore, the method was refined and a relatively less conservative parameter that would simply choose the BLAST “top hit” (e = 0.0001) was selected as the most reliable approach to reflect the trypanosome diversity and sequence abundance within the samples.

To exclude sampling depth heterogeneity, alpha-rarefaction plots were generated in QIIME (rarefaction was set at 14,136 and 11,158 sequence reads for koalas and ticks, respectively). The graphs suggested that a satisfactory sampling depth had been obtained to accurately represent the trypanosome community present within the samples, as curves appeared to plateau after about 2,000 reads (data not shown).

NGS data obtained during the present study is available under NCBI BioProject ID: 2588872 (Accession No. PRJNA383324).

**Phylogenetic analysis**

Phylogenetic analysis was conducted on a selection of OTUs and 18S *Trypanosoma* reference sequences retrieved from GenBank. OTUs assigned to a species level that were representative of a relatively greater number of reads were selected; of which, one representative of each *Trypanosoma* sp. identified in the current study, from both koalas and ticks, were included in the phylogenetic analysis. All “unassigned” and “assigned to a genus-level only” OTUs were also added to the dataset for inspection.

Evolutionary analyses were conducted in MEGA 6 [33]. Sequences (n = 51) were aligned by CLUSTAL W [34] under the default settings. After global-trimming, the most appropriate nucleotide substitution model (i.e. with the lowest Bayesian information criterion—BIC score) was selected using the dedicated function (Find best DNA/protein model) in MEGA 6. The evolutionary history was inferred by Maximum Likelihood (ML) based on the Kimura 2-parameter model [35], using uniform rates and 95% partial deletion. The bootstrapping method (n = 500 replicates) was used to infer reliability for internal branch and the percentage (>60%) of trees, in which the associated taxa clustered together is shown next to the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All “unassigned” OTUs confirmed by phylogenetic analysis to be the result of non-specific amplifications were removed from the dataset, the analysis repeated and a final tree generated. Estimates of genetic distances between sequences were conducted using the Kimura 2-parameter model, in MEGA 6.
Trypanosoma 18S sequences of selected OTUs included in the phylogenetic analysis were submitted to GenBank under the following accession numbers: KX786142-KX786149 and KY640309-KY640322.

Sanger sequencing of the 18S rRNA gene
For Sanger sequencing, the internal forward and reverse primers S825F and S662R [28] used in the hemi-nested PCR, but without the MiSeq adapters, were used in a single-round PCR assay. The resultant amplicons were separated on a 2% agarose gel, excised and purified using an in-house filter tip method [36]. The gel products were then Sanger sequenced in both directions using the ABI Prism Terminator Cycle Sequencing kit (Applied Biosystems, USA), on an Applied Biosystem 3730 DNA Analyzer. The sequences were BLAST-searched online against the GenBank nucleotide database (https://blast.ncbi.nlm.nih.gov). The results provided by Sanger and NGS methods were compared.

Results
Sanger sequencing analysis
In total, 59 out of 168 koala blood samples (35.1%, 95% CI: 27.9–42.8%) were positive for Trypanosoma DNA by single-round PCR. Sanger sequencing of amplicons obtained from these 59 samples revealed the presence of three species: T. irwini, T. gillettii and T. copemani, at prevalences of 28.6% (95% CI: 21.9–36%), 1.2% (95% CI: 0.1–4.2%) and 5.4% (95% CI: 2.5–9.9%), respectively.

A total of 23 ticks were positive by single-round PCR, however specific Trypanosoma DNA sequences were only obtained for 21 of these samples by Sanger sequencing (23.1%, 95% CI: 14.9–33.1%). Only two species (T. gillettii and T. copemani) were identified within ticks using Sanger sequencing, at prevalences of 17.6% (95% CI: 10.4–27%) and 5.5% (95% CI: 1.8–12.4%), respectively. No amplicons were detected after the PCR of non-template control and extraction blank control.

NGS bioinformatics analysis
A total of 3,565,056 paired-end reads were obtained from 61 out of 175 (34.9%) koala blood samples (including the positive controls) and 23 out of 91 (25.3%) ticks. Of the total number of sequences, 3,475,280 (97.5%) contained the correct primer sequences (no SNPs) and were retained for further analyses. During the FASTQ filtering process, 97% of the reads were retained and 3% were excluded from the dataset due to their low quality. A total of 5,990 OTUs were created during the OTU clustering step, of which 92.8% passed chimera filtering. Taxonomy was assigned to 5,562 OTUs, whilst 15 OTUs had no BLAST hits.

Overall prevalence and molecular characterisation of Trypanosoma spp. by NGS
In total, 54 out of 168 koalas (32.2%, 95% CI: 25.2–39.8%) and 23 out of 91 ticks (25.3%, 95% CI: 16.7–35.5%) were positive for Trypanosoma spp. by NGS. The prevalence estimates of trypanosomes in I. holocyclus (34.3%, 95% CI: 19.1–52.2%) and I. tasmani (19.7%, 95% CI: 10.2–32.4%) were not significantly different (p>0.05). Trypanosoma infection profiling by NGS analysis revealed the presence of five Trypanosoma spp. (T. irwini, T. gillettii, T. copemani, T. vegrandis and T. noyesi) within koala blood samples. The same trypanosome species were detected in ticks, except for T. noyesi. Additionally, a novel species classified only as Trypanosoma sp. by NGS analysis was present in DNA samples from koalas, I. holocyclus and I.
All positive controls produced identical results to those obtained in previous studies which used cloning of PCR products and species-specific PCRs to identify mixed trypanosome infections [3, 16]. No trypanosome sequences were obtained from the non-template control and extraction blank controls.

Phylogenetic analysis performed on a subset of NGS OTUs, representative of a relatively greater number of sequences isolated from koalas and ticks, confirmed their molecular identity during the taxonomic assignment process (Fig 1). Phylogenetic reconstructions also revealed that the NGS method was able to discriminate distinct genotypes of T. copemani (G1 and G2) and T. vegrandis (G5–G7 and AP-2011b-28 clone 11). The pairwise identity match between OTUs and their corresponding reference sequences (i.e. species they were identified as) ranged from 97% to 100%, according to the estimates of genetic divergence matrix (data not shown). Genetic variations of up to 7% within genotypes of the same species were observed.

OTUs assigned to a genus-level only in the NGS pipeline (hereafter referred as Trypanosoma sp. AB-2017), clustered together and formed a separate clade strongly supported by bootstrap values (Fig 1). This clade formed a sister clade to the group consisting of T. copemani, T. gilletti and T. vegrandis. Minor genetic divergence (1–2%) was observed between the sequences within the novel clade.

Molecular prevalence and genetic diversity of Trypanosoma spp. in koalas and ticks

The prevalence of each trypanosome species detected by NGS in koalas and ticks (I. tasmani and I. holocyclus), is presented in Fig 2. In koalas, the prevalence of T. irwini (32.1%, 95% CI: 25.2–39.8%), T. gilletti (25%, 95% CI: 18.7–32.3%) and T. copemani (27.4%, 95% CI: 20.8–34.8%) was significantly higher compared to the prevalence of T. vegrandis (10.1%, 95% CI: 6.0–15.7%), T. noyesi (0.6%, 95% CI: 0–3.3%) and novel Trypanosoma sp. AB-2017 (4.8%, 95% CI: 2.1–9.2%). No significant difference was observed among prevalences of the three dominant species (p > 0.05).

Trypanosoma irwini, T. gilletti and T. copemani were also the three most frequent isolates in I. holocyclus, with prevalence estimates of (34.3%, 95% CI: 19.1–52.2%), (31.4%, 95% CI: 16.9–49.3%) and (34.3%, 95% CI: 19.1–52.2%), respectively. However, the prevalence of each Trypanosoma spp. isolated from ticks were not statistically different from each other (p > 0.05). In I. tasmani ticks, even though the prevalence estimates for T. gilletti and T. copemani (19.6%, 95% CI: 10.2–32.4%, each) were relatively higher, they were not significantly different from those of T. irwini and T. vegrandis (12.5%, 95% CI: 5.2–24.1%, each). No major differences were observed in the pattern of Trypanosoma spp. prevalence when comparing koalas to ticks (partially matched data) or the two tick species to each other (Fig 2).

The prevalence of each genotype of T. copemani and T. vegrandis identified in koalas and ticks was also calculated, considering the potential differences in pathogenicity and/or virulence that might exist between these distinct variants (Table 1). In koalas, the prevalence of sequences classified as T. copemani isolate Charlton was significantly higher than those of T. copemani G1 and G2 (p < 0.05). As for T. vegrandis, G5 and G6 were the most common genotypes found in koalas; of which G6 showed a significantly higher prevalence compared to G3, G4, G7, AP-2011b-4c6 and AP-2011b-28c11 (p < 0.05). In contrast, all genotypes of T. copemani and T. vegrandis were present in statistically similar prevalences within I. holocyclus and I. tasmani (p > 0.05).

Among all koalas positive for Trypanosoma DNA by NGS (n = 54, excluding the positive controls), 34 (63%) were females and 20 (37%) were males. Overall, no significant difference
between the prevalence of trypanosomes in koala females (39.1%, 95% CI: 28.8–50.1%) and males (24.7%, 95% CI: 15.8–35.5%) was observed. Trypanosoma DNA was only detected by NGS in female ticks; however, this result should be cautiously interpreted as the sample size of male ticks (n = 9) was considerably smaller than of females (n = 82).
Characterization of single and mixed trypanosome infections

Intra-individual trypanosome mixed infections were observed in koalas, *I. holocyclus* and *I. tasmani*, at relatively higher prevalences compared to infections involving a single species (Fig 3). Co-infections with up to five different species were observed in koalas and ticks, not considering the genotype diversity within *T. copemani* and *T. vegrandis*. The composition of these mixed infections, in relation to the number of different species involved, is shown in Fig 4.

### Table 1. Prevalence of known genotypes of *T. copemani* and *T. vegrandis* in koalas and ticks (*Ixodes holocyclus* and *Ixodes tasmani*), determined by NGS at the 18S rRNA locus.

<table>
<thead>
<tr>
<th>Trypanosome</th>
<th>Genotype (GenBank accession code)</th>
<th>Koala</th>
<th><em>I. holocyclus</em></th>
<th><em>I. tasmani</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. copemani</em></td>
<td>Charlton (GU966588)</td>
<td>27.4  (20.8–34.8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.2  (2.3–19.6)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.5  (4.4–17.2)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G1 (KC753530)</td>
<td>1.2   (0.1–4.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2  (3.4–22.2)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>8.4  (2.3–15.9)&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G2 (KC753531)</td>
<td>4.8   (2.1–9.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.4  (8.8–32)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.5  (4.4–17.2)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>T. vegrandis</em></td>
<td>G3 (KC753533)</td>
<td>0     (0–2.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–7.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–3.8)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G4 (KC753532)</td>
<td>0     (0–2.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–7.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–3.8)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G5 (KC753534)</td>
<td>4.2   (1.7–8.4)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>0     (0–7.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–3.8)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G6 (KC753535)</td>
<td>7.1   (3.7–12.1)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0     (0–7.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–3.8)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>G7 (KC753536)</td>
<td>0.6   (0–3.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–7.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–3.8)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AP-2011b-4c6 (JN315392)</td>
<td>0     (0–2.2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.2  (3.4–22.2)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.4  (3–14.6)&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<td></td>
<td>AP-2011b-28c11 (JN315387)</td>
<td>0.6   (0–3.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–7.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0     (0–3.8)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in the same column followed by different letters are statistically distinct (p<0.05)

https://doi.org/10.1371/journal.pone.0181279.t001
**Fig 3.** Prevalence of single and mixed trypanosome infections in koalas, *Ixodes holocyclus* and *Ixodes tasmani*, determined by NGS. Error bars represent 95% confidence intervals (95% CI).

https://doi.org/10.1371/journal.pone.0181279.g003

**Fig 4.** Characterisation of mixed trypanosome infections in koalas (A), *Ixodes holocyclus* (B) and *Ixodes tasmani* (C), based on the number of unique trypanosome species involved. Each doughnut chart shows proportions of the total number (n) of samples harbouring more than one *Trypanosoma* sp., based on the NGS analysis.

https://doi.org/10.1371/journal.pone.0181279.g004
Infections involving three and four *Trypanosoma* spp. were more frequent in koalas, whereas in ticks, the majority of samples with mixed infections, harboured four *trypanosome* species.

Most mixed infections with three species in koalas consisted of co-infections with *T. irwini*, *T. gilletti*, and *T. copemani* at a prevalence of 12.5% (95% CI: 7.9%-18.5%). Of all cases of mixed infections involving four *Trypanosoma* spp., coinfections involving *T. irwini*, *T. gilletti*, *T. copemani* and *T. vegrans* were the most prevalent (7.7%, 95% CI: 4.2–12.8%). *Trypanosoma irwini* was the parasite identified in all cases of single infection in koalas.

In *I. holocyclus*, cases of multiple infections involving *T. irwini*, *T. gilletti*, *T. copemani* and *T. vegrans* were the most prevalent (27.3%, 95% CI: 13.3%-45.5%). The majority of *I. tasmani* ticks that were positive for *Trypanosoma* DNA also harboured mixed infections with these four *trypanosome* species (prevalence: 12.2%, 95% CI: 4.6–24.8%); however, co-infections with *T. gilletti*, *T. copemani* and *T. vegrans* were relatively common among the positives (prevalence: 8.2%, 95% CI: 2.3%-19.9%).

### NGS coverage and *Trypanosoma* abundance

In total, 3,027,379 reads obtained from koala blood and ticks, using NGS, were identified as *Trypanosoma* spp. This total includes the OTUs assigned to a genus or species level and the sequences they clustered with during the OTU clustering step. Table 2 displays the total number and proportion of NGS reads assigned to each *Trypanosoma* sp. detected in koalas, *I. holocyclus* and *I. tasmani*. Overall, in koalas, a remarkably high proportion of the reads (79%) were identified as *T. irwini*. In contrast, over 99.8% of the sequences obtained from *I. holocyclus* were taxonomically assigned as *T. gilletti*. A high number of copies identified as *T. gilletti* and *T. copemani* were isolated from *I. tasmani*, which represented 50% and 46% of the total, respectively. The proportion of sequences identified as *T. irwini* in both tick species investigated (up to 3.1% only) was significantly lower compared to koalas (p<0.05).

The NGS data analysis revealed that *T. vegrans*, *Trypanosoma* sp. AB-2017 and *T. noyesi* (detected in koalas only) produced a considerably lower amount of sequence reads overall compared to *T. irwini*, *T. gilletti* and *T. copemani*. However, analysis of sequence profile per sample revealed that these presumably rare species occurred in more than one sample, with corresponding number of reads ranging from 12 to 9,463, per sample. This provides supporting evidence that the reads were true sequences (i.e. not originated from possible sequencing errors).

To examine intra-individual genetic variation of the parasites, excluding the sampling depth heterogeneity, a composition plot was generated from rarefied data to represent the

<table>
<thead>
<tr>
<th>Trypanosoma spp.</th>
<th>Koala (n = 61*)</th>
<th><em>I. holocyclus</em> (n = 12)</th>
<th><em>I. tasmani</em> (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of sequences</td>
<td>%</td>
<td>Number of sequences</td>
</tr>
<tr>
<td><em>T. irwini</em></td>
<td>2,052,257</td>
<td>79.702</td>
<td>87</td>
</tr>
<tr>
<td><em>T. gilletti</em></td>
<td>254,721</td>
<td>9.892</td>
<td>234,427</td>
</tr>
<tr>
<td><em>T. copemani</em></td>
<td>251,770</td>
<td>9.778</td>
<td>164</td>
</tr>
<tr>
<td><em>T. vegrans</em></td>
<td>9,897</td>
<td>0.384</td>
<td>72</td>
</tr>
<tr>
<td><em>Trypanosoma</em> sp. AB-2017</td>
<td>6,247</td>
<td>0.243</td>
<td>26</td>
</tr>
<tr>
<td><em>T. noyesi</em></td>
<td>36</td>
<td>0.001</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>2,574,928</td>
<td>100</td>
<td>234,776</td>
</tr>
</tbody>
</table>

*54 positive samples + 7 positive controls

https://doi.org/10.1371/journal.pone.0181279.t002
relative proportion of multiple genotypes circulating within each positive koala sample (S1 Fig) and tick (S2 Fig). The analysis shows that 100% of the koalas positive for *Trypanosoma* DNA were infected with *T. irwini*, and also confirms the relatively higher abundance of *T. irwini* sequences within most koala samples positive by NGS. The results also show a relatively high frequency of *T. gillettii* in ticks, which was the dominant trypanosome species within the invertebrate hosts.

### Comparison of NGS and Sanger sequencing-based assay performances

A comparison between prevalence estimates of *Trypanosoma* spp. in koalas and ticks, obtained using NGS and Sanger sequencing is presented in Table 3.

Although the overall prevalence of *Trypanosoma* in koalas obtained using the Sanger sequencing method (35.1%, 95% CI: 27.9–42.8%) was slightly higher than the prevalence obtained by NGS (32.2%, 95% CI: 25.2–39.8%), the specific prevalences of *T. gillettii* and *T. copemani*, were significantly underestimated by Sanger sequencing when compared to results obtained using NGS (p<0.05). Sanger sequencing also failed to identify *T. ve grandi s*, *T. noyesi* and novel *Trypanosoma* sp. AB-2017 in koala blood samples.

In ticks, although the same number of positives (n = 23) was obtained by hemi-nested and single-round PCR, the overall prevalence of *Trypanosoma* sp. by NGS was slightly higher compared to that obtained using Sanger sequencing (23.1%, 95% CI: 14.9–33.1%), given that two PCR-positive samples were not confirmed to be *Trypanosoma* DNA using the latter approach. The prevalence of *T. copemani* in ticks estimated from Sanger sequencing results was significantly lower when compared to those obtained by NGS (p<0.05). Sanger sequencing was unable to detect *T. irwini*, *T. ve grandi s* and novel *Trypanosoma* sp. AB-2017 in ticks.

In most cases of trypanosomes isolated from koala blood samples and ticks (97% and 91%, respectively), Sanger sequencing-based identifications corresponded to the most abundant species (i.e. species with greater amount of reads within the sample) identified by NGS (S2 Table and S3 Table, respectively). In addition, a total of 42 (51.2%) clean and 40 (48.8%) mixed chromatograms were obtained, which differs considerably from the proportion of single and mixed infections in both koalas and ticks, determined by NGS (Fig 3). This indicates that mixed chromatograms were not necessarily obtained in all cases of trypanosome co-infections.

### Table 3. Comparison of prevalence estimates of *Trypanosoma* spp. obtained using NGS and Sanger sequencing in koalas and ticks (*Ixodes holo cyclus* and *Ixodes tasmani*).

<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Koala</th>
<th><em>I. holocyclus</em></th>
<th><em>I. tasmani</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevalence % (95%CI)</td>
<td>Prevalence % (95%CI)</td>
<td>Prevalence % (95%CI)</td>
</tr>
<tr>
<td></td>
<td>NGS</td>
<td>Sanger</td>
<td>NGS</td>
</tr>
<tr>
<td><em>T. irwini</em></td>
<td>32.1 (25.2–39.8)</td>
<td>28.6 (21.9–36)</td>
<td>34.3 (19.1–52.2)*</td>
</tr>
<tr>
<td><em>T. gillettii</em></td>
<td>25 (18.7–32.3)*</td>
<td>1.2 (0.1–4.2)</td>
<td>31.4 (16.9–49.3)</td>
</tr>
<tr>
<td><em>T. copemani</em></td>
<td>27.4 (20.8–34.8)*</td>
<td>5.4 (2.5–9.9)</td>
<td>34.3 (19.1–52.2)*</td>
</tr>
<tr>
<td><em>T. ve grandi s</em></td>
<td>10.1 (6.0–15.7)*</td>
<td>0 (0–2.2)</td>
<td>14.3 (4.8–30.3)</td>
</tr>
<tr>
<td><em>Trypanosoma</em> sp. AB-2017</td>
<td>4.8 (2.1–9.2)</td>
<td>0 (0–2.2)</td>
<td>8.6 (1.8–23.1)</td>
</tr>
<tr>
<td><em>T. noyesi</em></td>
<td>0.6 (0–3.3)</td>
<td>0 (0–2.2)</td>
<td>0 (0–10)</td>
</tr>
</tbody>
</table>

*An asterisk indicates that the prevalence obtained by NGS is significantly higher than the prevalence obtained using Sanger sequencing (p<0.05), for that particular trypanosome and host species.

https://doi.org/10.1371/journal.pone.0181279.t003
Trypanosoma infection profile of blood samples and ticks concurrently collected from the same koala host

In seven out of 15 cases in which tick and blood sample were concurrently collected from the same koala, both samples were negative for Trypanosoma DNA. In another seven cases, ticks removed from positive koalas were negative for Trypanosoma DNA. Interestingly, the trypanosome species characterized from a blood sample from one koala (T. irwini), was different from that of the tick (I. tasmani) (T. gilletti) collected from the same koala, as determined by Sanger sequencing. NGS analysis identified T. irwini (17,713 reads), T. copemani (18 reads), T. gilletti (5 reads) and T. noyesi (3 reads) in the koala blood sample; whereas only T. gilletti (33,982 reads) and T. copemani (7 reads) were identified in the tick.

Discussion

The application of NGS metabarcoding techniques can greatly improve our knowledge about the biodiversity of trypanosomes infecting Australian wildlife. This is the first study to successfully establish an NGS-based methodology, to audit trypanosome communities harboured by koalas and ticks. Notably the new method proved to be an efficient approach to characterise trypanosome polyparasitism, allowing identification of less abundant genotypes/species that are overlooked by Sanger sequencing alone.

The use of hemi-nested and not single-round PCR prior to NGS analysis constitutes a limitation of the present research, as nested PCR approaches involve an inherent risk of contamination and amplification biases [37]. However, this approach was necessary to overcome the reduction in sensitivity that the addition of MiSeq adapters to the primers had on amplification efficiency, as reported in previous studies [22, 23, 25].

To date, research using NGS platforms to investigate eukaryotic parasites have mainly focused on zoonotic pathogens [22, 24, 38–45]; hence, high-throughput methods to investigate protozoan parasitic diseases in wildlife are not yet well-established [25, 46–48]. More recently, an NGS pipeline was developed to analyse microbial eukaryotic communities, using Eimeria sp. as a model [25]. Although the method provides useful tools and insights, a broader use of an experiment designed specifically for Eimeria sp. may be problematic. This is because bioinformatics algorithms and similarity thresholds must be carefully selected and adjusted for Trypanosoma studies, relying on background knowledge of this parasite’s phylogeny and epidemiology. The present NGS-based assay, although performed in koala blood samples and ticks, may also prove useful in future investigations of mixed trypanosome infections in a range of other invertebrate and vertebrate hosts, including domestic animals and humans. For each case, an optimisation of PCR amplification conditions may be necessary.

The reliability of the novel methodology was assessed based on several criteria. For instance, all positive controls harbouring dual or triple mixed trypanosome infections with T. irwini, T. gilletti and T. copemani, which had previously been identified by Sanger sequencing of cloned PCR products or species-specific PCRs [3, 16], produced identical results by NGS in the present study. The assay also successfully identified the additional positive control, T. cruzi, which is relevant as surveillance of zoonotic pathogens in native species is a current research priority [49–51].

Phylogenetic analysis confirmed the classification of representative OTUs identified as Trypanosoma sp. in the pipeline, and demonstrated a high diversity between and within koala-derived trypanosomes. The analysis also indicated the phylogenetic position of the novel Trypanosoma sp. clade, which was strongly supported by bootstrap values and formed a sister clade to the group consisting of T. copemani, T. gilletti and T. vegrandis. Unfortunately, the relatively short length of amplicons, required for MiSeq sequencing, limits further inferences on
the potential novel species' genetic characterisation. Molecular analyses targeting additional loci and longer amplicons using Sanger sequencing are required for species delimitation in trypanosomes [52]. Hence, we recommend the use of NGS as the first step to investigate trypanosome genetic diversity, which should be followed by additional Sanger sequencing of longer amplicons for reliable phylogenetic characterization of any novel species identified.

NGS analysis of koala blood samples indicated that *T. irwini*, *T. copemani* and *T. gilletti* were the predominant trypanosome species in this marsupial, which is in line with previous findings based on Sanger sequencing [3, 16]. *Trypanosoma irwini*, besides being the most prevalent, was also the species which produced the greatest amount of sequences overall within most samples. Unfortunately, a critical challenge that limits interpretations related to NGS quantitative analyses (i.e. *Trypanosoma* abundance), is that the number of sequences generated from each variant by NGS may not reflect the number of microorganisms originally present in the sample. This is due to PCR bias (which may skew OTUs relative abundance) and to a possible variation in 18S rRNA gene copy numbers across the trypanosome species identified [53–55].

*Trypanosoma vегrandis*, *T. noyesi* (identified for the first time in koalas) and the novel *Trypanosoma* sp. AB-2017 OTU were found at a relatively low prevalence in koala hosts, and generated considerably less sequences compared to other co-infecting variants within koala samples such as *T. irwini*, *T. gilletti* and *T. copemani*. This may be the reason why these three species have not yet been detected in koalas using *Trypanosoma* sp. generic primers and Sanger sequencing. *Trypanosoma vегrandis* has only recently been isolated from koalas; and this was only possible after morphological visualization of the parasite, which led to the use of a *T. vегrandis*-specific assay for molecular detection [17]. However, discoveries like this may be difficult for novel species or species with low levels of parasitaemia, which is the case of *T. noyesi* [50].

Little is known about the clinical significance of *Trypanosoma* spp. infecting koalas. However, it is noteworthy that *T. gilletti* has been implicated in the decreased survival of koalas with signs of concomitant diseases; and *T. copemani* has been associated with anaemia in this marsupial [3]. Additionally, *T. copemani* has been associated with pathological changes such as muscle degeneration in woylies (*Bettongia penicillata ogilbyi*) [4, 5]. The genetic proximity of *T. noyesi* to the pathogenic *T. cruzi* is of potential concern from a conservation and public health perspective; however, intracellular stages of *T. noyesi*, suggestive of trypanosome pathogenicity, have not been observed in woylies [50].

Importantly, prevalences of single and mixed trypanosome infections in koalas, obtained by NGS (4.8% and 27.4% respectively), were remarkably different from those obtained by cloning associated with Sanger sequencing (51.1% and 1.5% respectively) [16]. A possible explanation is that reliance on mixed sequencing chromatograms as indicative of multiple trypanosome infections, broadly used as a selection criteria for cloning, may be inaccurate, as demonstrated by our results.

This is the first report of trypanosome polyparasitism involving up to five species. Multiple trypanosome infections involving up to 3 and 4 genotypes have been reported in koalas and woylies, respectively [3–5, 16, 17, 21]. Co-infections composed of *T. irwini*, *T. gilletti* and *T. copemani* were the most frequent among the koala populations sampled, followed by concomitant infections with *T. irwini*, *T. gilletti* and *T. copemani*. Our results differ from previous observations that co-infections involving only *T. irwini* and *T. gilletti* were predominant among koalas, but are consistent with the report of *T. copemani* being more common as a triple co-infection with *T. irwini* [3].

The implications of interactions amongst multiple trypanosomes in a single vertebrate host by either reducing or enhancing parasitaemia, virulence or pathogenicity are still unclear [51, 56, 57]. A previous molecular survey identified mixed infections involving *T. vегrandis*, *T.
Next-generation sequencing approach to characterize mixed trypanosome infections in koalas and ixodes ticks

copemani and T. noyesi at a higher prevalence in a declining woylie population compared to a stable population [4]. In contrast, another study suggested there may be an interspecific competition between T. copemani and T. vegrandsis in woylies, whereby T. vegrandsis may moderate the sequential establishment of T. copemani [5]. Further investigations are essential to elucidate whether trypanosome polyparasitism may aggravate the consequences of infection; and to quantify the contribution of trypanosome infection, in single and mixed infections, (co-infections with Chlamydia and KoRV) to the koala population decline.

NGS revealed a high trypanosome genetic diversity within I. holocyclus and I. tasmani, with five species (T. irwini, T. gilletti, T. copemani, T. vegrandsis and novel Trypanosoma sp. AB-2017) identified within these hosts. In contrast to NGS of koalas, in both tick species examined by NGS, T. gilletti was the dominant species, with the proportion of T. irwini sequences (up to 3.1%) being unexpectedly significantly lower than in koalas. However, although koalas and ticks investigated are from the same region of Australia, the fact that they constituted independent sampling groups (i.e. only a small proportion of ticks and blood samples were concurrently collected from the same koala), makes it difficult to make inferences about the host-vector-parasite relationships.

The differences in the trypanosome species identified in a koala blood sample and a tick removed from the same animal is interesting. NGS identified T. irwini in >99.8% of sequences from the koala blood sample, whereas T. gilletti was the dominant species identified in the tick (which was also the dominant species in ticks for which a corresponding koala blood sample was not available). This suggests that the trypanosome DNA detected within the tick, was unlikely to have originated from the koala blood meal. However, it is important to note that the number of sequences assigned to each Trypanosoma sp. by NGS does not necessarily reflect the actual parasite numbers originally present within the sample, due to possible amplification bias and because the 18S rRNA copy numbers of the different Trypanosome species identified in the present study (which are currently unknown) may vary. In addition, low abundance trypanosome species in the tick may still be transmitted, particularly if they are capable of multiplying/differentiating in the vector (cyclical transmission) or if able to survive in the tick’s digestive tract until it is accidentally ingested by another host (mechanical transmission). Further research on a larger number of koala blood samples and corresponding ticks and the fate of these trypanosomes in ticks is required to better understand the role of ticks in the transmission of different trypanosome species to koalas.

This is the first report of trypanosome polyparasitism in Australian native ticks, which is probably due to limitations of Sanger sequencing used in previous surveys. It is possible that, in each tick species, the most prevalent and abundant Trypanosoma spp. is transmitted by the tick. On the other hand, it may also be possible that ticks can individually be vectors for more than one Trypanosoma spp. Further research is required to clarify the vectorial role of Ixodes ticks in Trypanosoma spp. transmission.

The identification of trypanosomes in an ectoparasite does not make it a vector, as the DNA detected could represent ingested parasites from a blood meal. However, mechanical transmission where the parasite can survive in the vectors’ digestive tract and then be passed on to another host cannot be ruled out [49, 51]. Cross-transmission experiments from infected vector candidates (ticks) to uninfected mammalian host have previously been used to confirm vectors [49]. However this is often impractical and in the case of threatened marsupials such as the koala, it is unethical.

Based on parasite detection only, I. australiensis has been identified as a potential vector for T. copemani [58]; and tabanid flies (family Tabanidae) and biting midges (family Ceratopogonidae), have been suggested as candidate vectors for T. noyesi [50]. In the present study, NGS of ticks identified four koala-derived Trypanosoma spp. within I. tasmani and I. holocyclus. To the best of our knowledge, this is the first identification of I. tasmani as a potential vector for
Trypanosoma spp. A previous study relying on morphological tools only has found trypanosomes in an I. holocyclus tick retrieved from a bandicoot positive for T. thylacis [59]; thus this is the first identification of T. irwini, T. gilletti, T. copemani, and T. vegrandis in I. holocyclus, which is based on molecular evidence.

The finding of I. holocyclus harbouring T. copemani could potentially be of public health significance, given the natural resistance of T. copemani to human serum [60]. Nevertheless, further research is required to determine the zoonotic potential of T. copemani and other koala-derived trypanosomes. From a One Health perspective, it is essential to monitor the presence of trypanosomes in wildlife and their ectoparasites using NGS, considering their potential to harbour zoonotic pathogens [61].

Conclusions

In conclusion, the novel NGS-based method for the detection and characterisation of Trypanosoma, described in the present study, constitutes an efficient molecular tool to audit the genetic diversity of trypanosomes in koalas and candidate vectors. Our results highlight the greater accuracy of NGS compared to Sanger sequencing, as the latter clearly underestimated the prevalence of rare isolates within the samples examined; and overlooked the presence of novel species. Therefore, Sanger sequencing of PCR amplicons obtained using Trypanosoma generic primers is ineffective for the detection of mixed trypanosome infections in native species.

Next-generation sequencing analysis provided new insights into trypanosomes genetic diversity in koalas and identified, for the first time, two species of native ticks (I. holocyclus and I. tasmani) as vector candidates for T. gilletti, T. irwini, T. copemani, T. vegrandis and a novel koala-derived trypanosome. The discovery that mixed infections with up to five Trypanosoma spp. are significantly more prevalent than single trypanosome infections in koalas, constitutes a benchmark for future clinical and epidemiological studies required to quantify the contribution of trypanosomes on clinical disease in koalas, particularly in the presence of concurrent pathogens such as Chlamydia and KoRV. Such knowledge can support important decisions on koala health and population management, helping to stabilize population decline events.

Supporting information

S1 Fig. Composition plot illustrating the trypanosome infection profile of each positive koala sample at the 18S rRNA locus, by NGS. Rarefaction was set at 14,136 sequences. (TIF)

S2 Fig. Composition plot illustrating the trypanosome infection profile of each positive tick sample at the 18S rRNA locus, by NGS. Rarefaction was set at 11,158 sequences. (TIF)

S1 Table. GenBank accession numbers of Trypanosoma 18S rDNA sequences used as reference for NGS taxonomic assignment and phylogenetic analysis. (DOCX)

S2 Table. Taxonomic characterisation of trypanosome-specific sequences obtained from koalas, by NGS and Sanger sequencing at the 18S rRNA locus. Values represent the number of sequences obtained by NGS, from each sample. Genbank codes correspond to the sequences’ closest matches. (DOCX)

S3 Table. Taxonomic characterisation of trypanosome-specific sequences obtained from ticks, by NGS and Sanger sequencing at the 18S rRNA locus. Values represent the number
of sequences obtained by NGS, from each sample. Genbank codes correspond to the sequences’ closest matches.

Acknowledgments

The authors wish to thank Ms. Cheyne Flanagan from The Koala Hospital, Ms. Joanne Loader from Endeavour Veterinary Ecology Pty and the staff of AZWH for their assistance with sample collection. Authors would also like to acknowledge Dr. Linda McInnes, who kindly provided the koala blood samples used as positive controls; Ms. Sarah Keatley for providing a DNA isolate from Trypanosoma cruzi; and Dr. Sam Abraham and Ms. Frances Briggs, from Western Australian State Agricultural Biotechnology Centre (SABC), for their support.

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Formal analysis: Amanda D. Barbosa, Andrea Paparini.


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Project administration: Una Ryan.

Resources: Amber Gillett, Kristin Warren, Peter Irwin, Una Ryan.

Supervision: Kristin Warren, Peter Irwin, Una Ryan.

Validation: Amanda D. Barbosa, Alexander W. Gofton, Andrea Paparini.

Visualization: Amanda D. Barbosa.

Writing – original draft: Amanda D. Barbosa.

Writing – review & editing: Amanda D. Barbosa, Alexander W. Gofton, Andrea Paparini, Annachiara Codello, Telleasha Greay, Amber Gillett, Kristin Warren, Peter Irwin, Una Ryan.

References


Appendix E: Taxonomic characterisation of trypanosome-specific sequences obtained from koalas, by NGS and Sanger sequencing at the 18S rRNA locus. Values represent the number of sequences obtained by NGS, from each sample. Genbank codes correspond to the sequences’ closest matches.
<table>
<thead>
<tr>
<th>Sample ID</th>
<th>( T. irwini ) (FJ649479)</th>
<th>( T. gilletti ) (GU966589)</th>
<th>( T. copemani ) (GU966588; KC753530-32)</th>
<th>( T. vegrandis ) (KC753534-36; JN315387)</th>
<th>( T. noyesi ) -H25 (AJ009168)</th>
<th>( T. sp. AB-17 )</th>
<th>Total assigned</th>
<th>Sanger sequencing</th>
<th>Sanger sequencing (GenBank match)</th>
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<tr>
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Appendix F: Taxonomic characterisation of trypanosome-specific sequences obtained from ticks, by NGS and Sanger sequencing at the 18S rRNA locus. Values represent the number of sequences obtained by NGS, from each sample. Genbank codes correspond to the sequences’ closest matches.
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Research paper

Trypanosoma teixeirae: A new species belonging to the T. cruzi clade causing trypanosomosis in an Australian little red flying fox (Pteropus scapulatus)

Amanda D. Barbosa, John T. Mackie, Robyn Stenner, Amber Gillett, Peter Irwin, Una Ryan

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Article history:
Received 30 November 2015
Received in revised form 26 April 2016
Accepted 1 May 2016

Keywords:
Trypanosoma teixeirae sp. n
Little red flying fox (Pteropus scapulatus)
Morphology
PCR
18S ribosomal RNA (rRNA)
Glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH)
Phylogeny

Abstract

Little is known about the genetic diversity and pathogenicity of trypanosomes in Australian bats. Recently, a novel trypanosome species was identified in an adult female little red flying fox (Pteropus scapulatus) with clinical and pathological evidence of trypanosomosis. The present study used morphology and molecular methods to demonstrate that this trypanosome is a distinct species and we propose the name Trypanosoma teixeirae sp. n. Morphological comparison showed that its circulating trypanomastigotes were significantly different from those of Trypanosoma pteropi and Trypanosoma hipposideri, two species previously described from Australian bats. Genetic information was not available for T. pteropi and T. hipposideri but phylogenetic analyses at the 18S ribosomal RNA (rRNA) and glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) loci indicated that T. teixeirae sp. n. was genetically distinct and clustered with other bat-derived trypanosome species within the Trypanosoma cruzi clade.

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1. Introduction

Bats (order Chiroptera) are reservoirs of numerous zoonotic pathogens including rabies, Australian bat lyssavirus, severe acute respiratory syndrome (SARS), Hendra virus, Nipah virus and Ebola virus (Wood et al., 2012). Trypanosomes are blood-borne flagellate protozoan parasites that can infect a wide range of vertebrate hosts including humans. Numerous trypanosome species have been identified in bats in Asia, Africa, South America and Europe (Hoare, 1972; Baker, 1973; Marinkelle, 1976, 1979; Gardner and Molyneux, 1988a,b; Hamanaka and Pinto Ada, 1993; Steindel et al., 1998; Barnabe et al., 2003; Grisard et al., 2003; Lisboa et al., 2008; Cottontail et al., 2009; Maia da Silva et al., 2009; Cavazzana et al., 2010; Garcia et al., 2012; Hamilton et al., 2012; Lima et al., 2012, 2013; Marcili et al., 2013; Silva-Iturralde et al., 2013; Cottontail et al., 2014; Ramirez et al., 2014).

In Australia, three Trypanosoma spp. have been described in bats to date: Trypanosoma pteropi from the black flying fox (Pteropus gouldii) (Breinl, 1913; Mackerras, 1959). Trypanosoma hipposideri from the dusky horseshoe bat (Hipposideros bicolor albanensis) and Trypanosoma vegrantis, in pteropid bats (Yangochiroptera) and microbats (Yinpterochiroptera) (Austen et al., 2015). None of these have been associated with clinical disease. In addition, Mackie et al. (2015) recently described the first case of trypanosomosis in a little red flying fox (Pteropus scapulatus—suborder Yinpterochiroptera) from eastern Australia, caused by an apparently novel trypanosome species.

Molecular and phylogenetic studies have suggested that bat trypanosomes are implicated in the evolutionary history of the T. cruzi clade and may potentially be the precursor of trypanosomes from Australian marsupials and several African terrestrial mammals (Hamilton et al., 2012; Lima et al., 2013). There is however very limited knowledge about the genetic diversity of Australian bat trypanosomes, where only 9 of 76 indigenous bat species have been screened for this parasite (Thompson et al., 2014).

In the present study, we describe the morphological and genetic characterisation of the novel trypanosome in the little red flying fox
Table 1
Genbank accession numbers and sources (where known) of trypanosome isolates included in the phylogenetic analyses.

<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Host origin</th>
<th>Geographic origin</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. teixeirae sp. n.</td>
<td>Bat (Pteropus scapulatus)</td>
<td>Australia</td>
<td>KT907061 KT907062</td>
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<tr>
<td>T. rangeli</td>
<td>Bat (Platyrrhinus lineatus)</td>
<td>Brazil</td>
<td>FJ00242 FJ0140364</td>
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<tr>
<td>T. minasense</td>
<td>Tamarin (Saguinus midas)</td>
<td>Japan</td>
<td>AJ012413 AB362561</td>
</tr>
<tr>
<td>T. dionisi</td>
<td>Bat (Eptesicus brasilensis)</td>
<td>Brazil</td>
<td>FJ001666 GJ0140362</td>
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<tr>
<td>T. erneyi</td>
<td>Bat (Tadarida sp.)</td>
<td>Mozambique</td>
<td>JN040987 JN040964</td>
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<tr>
<td>T. vespertilionis</td>
<td>Bat (Pipistrellus pipistrellus)</td>
<td>England</td>
<td>AJ009166 A620283</td>
</tr>
<tr>
<td>T. sp. bat</td>
<td>Bat (Rousettus aegyptiacus)</td>
<td>Gabon</td>
<td>A620283</td>
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<tr>
<td>T. livingstonei TCC1270</td>
<td>Bat (Rhinolophus landeri)</td>
<td>Mozambique</td>
<td>KF192979 KF192958</td>
</tr>
<tr>
<td>T. livingstonei TCC1953</td>
<td>Bat (Hipposideros caffer)</td>
<td>Mozambique</td>
<td>– KF192969</td>
</tr>
<tr>
<td>T. cruzi Tcbat</td>
<td>Bat (Myotis levis)</td>
<td>Brazil</td>
<td>FJ002421 GQ140358</td>
</tr>
<tr>
<td>T. cruzi marinkellei</td>
<td>Bat (Carollia perspicillata)</td>
<td>Brazil</td>
<td>FJ001664 GQ140360</td>
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<tr>
<td>T. cruzi Y</td>
<td>Human (Homo sapiens)</td>
<td>Brazil</td>
<td>AF301912 GQ140353</td>
</tr>
<tr>
<td>T. conorhini</td>
<td>Rat (Rattus rattus)</td>
<td>Australia</td>
<td>AJ012411 A620267</td>
</tr>
<tr>
<td>T. sp. Nan Doum1</td>
<td>Palm civet (Nandinia bintorata)</td>
<td>Cameroon</td>
<td>FM202492 FM164793</td>
</tr>
<tr>
<td>T. sp. Hoch Ndi1</td>
<td>Monkey (Cercopithecus nictitans)</td>
<td>Cameroon</td>
<td>FM202493 FM164794</td>
</tr>
<tr>
<td>T. sp. H25</td>
<td>Kangaroo (Macropus giganteus)</td>
<td>Australia</td>
<td>AJ009168 A620276</td>
</tr>
<tr>
<td>T. sp. AP-2011-64</td>
<td>Possum (Trichosurus vulpecula)</td>
<td>Australia</td>
<td>JN315383 A620276</td>
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<tr>
<td>T. sp. AB-2013-G8</td>
<td>Woylie (Bettongia penicillata)</td>
<td>Australia</td>
<td>KC753537 K612988</td>
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<tr>
<td>T. aivium</td>
<td>Eagle (Aquila pomarina)</td>
<td>Slovakia</td>
<td>AF416559 –</td>
</tr>
<tr>
<td>T. sp. AAT</td>
<td>Currawong (Strepera sp.)</td>
<td>Australia</td>
<td>AJ620557 A620264</td>
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<tr>
<td>T. bennetti</td>
<td>American kestrel (Falco sparverius)</td>
<td>Germany</td>
<td>AF235562 FJ648946</td>
</tr>
<tr>
<td>T. irwini</td>
<td>Koala (Phascolarctos cinereus)</td>
<td>Australia</td>
<td>FJ649479 FJ649485</td>
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<tr>
<td>T. lewisii</td>
<td>Rat (Rattus rattus)</td>
<td>England</td>
<td>AJ009156 A620272</td>
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<tr>
<td>T. microti</td>
<td>Vole (Microtus agrestis)</td>
<td>England</td>
<td>AJ009158 A620273</td>
</tr>
<tr>
<td>T. vivax</td>
<td>Cattle</td>
<td>EU477537 AF053744</td>
<td></td>
</tr>
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<td>T. brucei brucei</td>
<td>Human (Homo sapiens)</td>
<td>Uganda</td>
<td>AJ009142 –</td>
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<td>Human (Homo sapiens)</td>
<td>Nigeria</td>
<td>AJ009141 –</td>
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<td>T. evansi</td>
<td>Capybara (H. hydrochaeris)</td>
<td>Brazil</td>
<td>AJ009154 AF053743</td>
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<tr>
<td>T. copomani Charlton</td>
<td>Koala (Phascolarctos cinereus)</td>
<td>Australia</td>
<td>GJ966588 –</td>
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<tr>
<td>T. capomani Mika</td>
<td>Koala (Phascolarctos cinereus)</td>
<td>Australia</td>
<td>GJ966585 –</td>
</tr>
<tr>
<td>T. capomani G1</td>
<td>Woylie (Bettongia penicillata)</td>
<td>Australia</td>
<td>K612982</td>
</tr>
<tr>
<td>T. capomani G2</td>
<td>Woylie (Bettongia penicillata)</td>
<td>Australia</td>
<td>K612983</td>
</tr>
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<td>T. gilletti</td>
<td>Koala (Phascolarctos cinereus)</td>
<td>Australia</td>
<td>GJ966589 GJ966587</td>
</tr>
<tr>
<td>T. vehrhardis G3</td>
<td>Woylie (Bettongia penicillata)</td>
<td>Australia</td>
<td>K612984</td>
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<td>T. vehrhardis G4</td>
<td>Woylie (Bettongia penicillata)</td>
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<td>K612985</td>
</tr>
<tr>
<td>T. vehrhardis G5</td>
<td>Woylie (Bettongia penicillata)</td>
<td>Australia</td>
<td>K612986</td>
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<tr>
<td>T. vehrhardis G6</td>
<td>Woylie (Bettongia penicillata)</td>
<td>Australia</td>
<td>K612987</td>
</tr>
<tr>
<td>T. vehrhardis G7</td>
<td>Woylie (Bettongia penicillata)</td>
<td>Australia</td>
<td>K612989</td>
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<tr>
<td>T. major</td>
<td>African toad (Bufo regularis)</td>
<td>Africa</td>
<td>AJ009157 A620253</td>
</tr>
<tr>
<td>T. rotatorium</td>
<td>Bullfrog (Rana catesbeiana)</td>
<td>Canada</td>
<td>AJ009161 A620256</td>
</tr>
<tr>
<td>T. binneyi</td>
<td>Platypus (Ornithorhynchus anatinus)</td>
<td>Australia</td>
<td>AJ132351 A620266</td>
</tr>
<tr>
<td>T. granulosum</td>
<td>Eel (Anguilla anguilla)</td>
<td>Portugal</td>
<td>AJ620552 –</td>
</tr>
<tr>
<td>T. sp. CLAR</td>
<td>Catfish (Clarias angolensis)</td>
<td>Africa</td>
<td>AJ620555 A620251</td>
</tr>
</tbody>
</table>

(Mackie et al., 2015), for which we proposed the name Trypanosoma teixeirae sp. n.

2. Material and methods

2.1. Sample collection

A venous blood sample was collected from the cephalic vein of an adult female little red flying fox that presented to the Australia Zoo Wildlife Hospital (AZWH) in April, 2014. The flying fox had been rescued from the ground at Redcliffe in south-eastern Queensland, Australia and was moribund with anaemia and icterus. Clinical and pathological evidence of disease consistent with trypanosomiasis in this flying fox was described by Mackie et al. (2015).

2.2. Morphological analyses

Thin blood smears were made from a drop of fresh blood and stained with Diff Quick (Siemens, Germany). After air-drying, the slides were then cover-slipped using DePeX mounting medium Gurr (Merck Pty. Limited, Kilsyth, Victoria, Australia). Stained films were systematically examined using a BX50 microscope (Olympus, Japan) with screen views generated by a DP Controller (version 3.2.1.276, Olympus, Japan). Digital light micrograph images of any trypomastigotes observed were taken at ×1000 magnification.

Digital images of the organisms identified in the blood films were used to measure key morphological features such as total length (TL), width (W), posterior to kinetoplast (PK), kinetoplast to nucleus (KN), nucleus to anterior (NA) and free flagellum (FF), according to parameters described by Hoare (1972) and Mackerras (1959). Means and standard errors were calculated. The morphological measurements were taken using the software ImageJ (Abramoff et al., 2004).

As two trypanosome species have previously been described in Australian bats based on morphological analysis only (Breinl, 1913; Mackerras, 1959), morphometrics of the novel trypanosome was compared statistically with available measurements for T. pteropi and T. hippocideri. Mean values for each morphological feature were calculated for T. teixeirae sp. n. whilst median values of reported ranges were used as input data for T. pteropi and T. hippocideri, as means were not available in the bibliographical references. Statistical analyses were conducted using the same sample t-test, in the software PAST 1.43 (Hammer et al., 2001).
2.3. DNA extraction

Genomic DNA was extracted from 200 μl of whole blood, using the MasterPure Purification Kit (Epicentre Biotechnologies, USA). A DNA extraction blank (with sterile molecular-grade water instead of blood) was included in the extraction to exclude the contamination of reagents and consumables with DNA.

2.4. 18S rRNA and GAPDH amplification and sequencing

A nested PCR protocol using generic Trypanosoma sp. primers SLF, S762R, S823F and S662R (Maslov et al., 1996; McLnnes et al., 2009) was performed to amplify an approximately 900 bp fragment of the 18S rRNA gene, as previously described by McLnnes et al. (2009). The DNA sample was also amplified at the glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) gene using a heminested PCR protocol (McLnnes et al., 2009).

PCR products were run on a 2% agarose gel containing SYBR Safe Gel Stain (InVitrogen, USA), and visualized with a dark reader trans-illuminator (Clare Chemical Research, USA). The gel bands were purified using an in-house filter tip method as previously described (Yang et al., 2013). All controls (positive, negative and DNA extraction blank) produced appropriate PCR results.

The purified PCR products were sequenced using the corresponding internal reverse primers diluted at 3.2 picomoles with an ABI PrismTM Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA Analyzer.

2.5. Phylogenetic analysis

Nucleotide sequences obtained at both 18S rRNA and GAPDH loci were aligned with additional trypanosome sequences retrieved from GenBank (Table 1) by MUSCLE (Edgar, 2004) using the default settings. Ambiguous regions containing gaps or poorly aligned were removed by Gblocks (Castresana, 2000), available on the Phylogeny.fr platform (Dereeper et al., 2008), using low stringency parameters. The curated alignments were imported into MEGA 6 (Tamura et al., 2013) and the most appropriate nucleotide substitution model was selected using the dedicated function.

The evolutionary histories at both 18S rRNA and GAPDH genes were inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The gamma shape parameter was estimated directly from the data. Reliability for internal branch was assessed using the bootstrapping method (500 bootstrap replicates) and support values (>60%) indicated at the left of each node. The phylogenetic trees were drawn to scale, with branch lengths measured in the number of substitutions per site.

Estimates of genetic divergence between sequences were generated in MEGA 6 based on the Tamura-Nei algorithm, using uniform rates and a partial deletion of 95%.

3. Results

3.1. Microscopy and morphometric analysis of T. teixeirae sp. n.

A total of nine organisms morphologically consistent with a trypanosome were detected by light microscopy in blood films from the little red flying fox. The extracellular organisms were slender with tapered ends, with long free flagella and either an undeveloped or absent undulating membrane. A nearly central nucleus and a terminal small round deeply staining internal structure consistent with a kinetoplast were also observed (Fig. 1a-c). The trypomastigotes varied in length from 20.4 to 30.8 μm (average 25.9 μm) and in width from 1.3 to 2.3 μm (average 1.9 μm) (Table 2).

Among the nine long slender organisms observed, two were not true trypomastigotes as their kinetoplast was located at the very end of the posterior, what made it impossible to calculate the PK distance. In another instance, the trypomastigote’s free flagellum was apparently under a red blood cell, hence any measurements taken of FF or TL would have been inaccurate. We have therefore only measured what was feasible, which explains the divergence in the number of organisms measured for each morphological feature (Table 2).

Three flagellate round forms with a flagellum running round the organism about 90 degrees were also observed (Fig. 1d). Their body shape was consistent with a sphaeromastigote or a round epimastigote if their flagellar position was considered.

Morphometric analysis revealed that although the reported length and width ranges for the T. teixeirae sp. n. and T. pteropi overlap, the former was significantly longer and thinner than the latter (p<0.01) (Table 3). There was no significant difference between KN, NA and FF dimensions between T. teixeirae sp. n. and T. pteropi. In addition, T. hippocideri was significantly smaller than T. teixeirae sp. n. for TL and FF dimensions (p<0.01) (Table 3). No significant difference was observed for B, PK and KN between T. teixeirae sp. n. and T. hippocideri.

3.2. Sequence and phylogenetic analysis

Maximum Likelihood analysis at both the 18S rDNA and GAPDH loci (Figs. 2 and 3, respectively) produced concordant tree topologies and revealed that T. teixeirae sp. n. grouped with other trypanosomes belonging to the T. cruzi clade, including 7 bat-derived isolates (T. cruzi Tcbat, T. cruzi marinkellei, T. erneyi, T. dionisi, T. rangeli, T. vesperblionis and T. sp. AJ012418/GQ140365) and three isolates from Australian marsupials (T. sp. H25 from a kangaroo- AJ009168/AJ620276; T. sp. AB-2003-G8 from a woylie- KC753537/KC812988; and T. sp. AP-2011-64 from a brush-tailed possum – JN315383/AJ620276). The phylogenetic trees also corroborated the evolutionary relationships among all major trypanosome clades described in previous broader analyses. Nucleotide sequences were obtained at both loci for T. teixeirae sp. n. were submitted to GenBank under the following accession numbers: KT907061 and KT907062.

Estimates of evolutionary divergence between nucleotide sequences revealed that T. teixeirae sp. n. was genetically distinct but most closely related to T. minasense and T. rangeli (genetic distances of 1% at the 18S rDNA locus and 14–15% at the gGAPDH, respectively) (Table 4).

4. Species description

Species Name: Trypanosoma teixeirae sp. n. (Fig. 1)

Type host: Little red flying fox (Pteropus scapulatus).

Other hosts: Unknown

Type Locality: Redcliffe peninsula, Queensland, Australia.

Prevalence: Unknown

4.1. Morphology

T. teixeirae sp. n. trypomastigotes are on average 25.9 μm long and 1.9 μm wide, exhibiting a slender shape with tapered ends, a nearly central nucleus and a small terminal kinetoplast. Undulating membrane either absent or under-developed.

4.2. Etymology

The species is named T. teixeirae sp. n. in honour of Prof. Marta Maria Gerais Teixeira, from the University of Sao Paulo, who has
Fig. 1. Light photomicrographs of diff quick stained blood film showing Trypanosoma teixeirae sp. n. trypomastigotes in the blood of a red flying fox (Pteropus scapulatus) (a–c) and (d) Round epimastigote form. Scale bars represent 10 μm.

Table 2
Mean dimensions and standard errors (S.E.) of morphological features of Trypanosoma teixeirae sp.n. isolated from a little red flying fox’s blood.

<table>
<thead>
<tr>
<th>Feature*</th>
<th>No. of organisms measured</th>
<th>Observed range (μm)</th>
<th>Mean ± S.E. (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length</td>
<td>8</td>
<td>20.4–30.8</td>
<td>25.9 ± 1.2</td>
</tr>
<tr>
<td>Width</td>
<td>8</td>
<td>1.3–2.3</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>PK</td>
<td>7</td>
<td>1.5–2.4</td>
<td>2 ± 0.15</td>
</tr>
<tr>
<td>KN</td>
<td>9</td>
<td>3.3–6.2</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>NA</td>
<td>9</td>
<td>5.1–9.8</td>
<td>7.8 ± 0.5</td>
</tr>
<tr>
<td>FF</td>
<td>8</td>
<td>10.0–12.9</td>
<td>11.3 ± 0.4</td>
</tr>
</tbody>
</table>

*Total length: total body length measured along mid-line including free-flagellum.
Width: maximum width measured at nucleus level (undulating membrane included).
PK: distance between the posterior end and the kinetoplast.
KN: distance between the kinetoplast and posterior edge of the nucleus.
NA: distance between the anterior edge of the nucleus and the anterior end of the body.
FF: length of the free flagellum.

Table 3
Comparison between morphological dimensions of blood trypomastigotes of Trypanosoma teixeirae sp. n. with Trypanosoma pteropi and Trypanosoma hipposideri.

<table>
<thead>
<tr>
<th>Morphological Feature (μm)</th>
<th>Total length</th>
<th>Width</th>
<th>PK</th>
<th>KN</th>
<th>NA</th>
<th>FF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypanosoma teixeirae sp.n.</td>
<td>20.4–30.8</td>
<td>1.3–2.3</td>
<td>1.5–2.4</td>
<td>3.3–6.2</td>
<td>5.1–9.8</td>
<td>10.0–12.9</td>
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<tr>
<td>Trypanosoma pteropi</td>
<td>18.0–22.0</td>
<td>2.0–4.0</td>
<td>1.5–4.0</td>
<td>4.0–5.0</td>
<td>8.0–10.0</td>
<td>8.0–12.0</td>
</tr>
<tr>
<td>Trypanosoma hipposideri</td>
<td>10.5–13.0</td>
<td>1.5–2.0</td>
<td>1.0–2.5</td>
<td>4.0–6.0</td>
<td>1.5–5.0</td>
<td>4.0–8.0</td>
</tr>
</tbody>
</table>

Total length: total body length measured along mid-line including free-flagellum; Width: maximum width measured at nucleus level (undulating membrane included); PK: distance between the posterior end and the kinetoplast; KN: distance between the kinetoplast and posterior edge of the nucleus; NA: distance between the anterior edge of the nucleus and the anterior end of the body; FF: length of the free flagellum. Ranges given with mean for Trypanosoma teixeirae sp. n. As the mean dimensions were not available for Trypanosoma pteropi or Trypanosoma hipposideri the median value of the range is presented in the table and was used for statistical analysis. For each column, values followed by an asterisk are significantly different to the T. teixeirae sp.n. value (p<0.01).

greatly contributed to the biology and phylogeny of trypanosome species.

5. Discussion

In the present study, we have described T. teixeirae sp. n., the causative agent of trypanosomosis in an Australian little red fly-
Fig. 2. Phylogenetic relationships of Trypanosoma teixeirae sp. n. with other trypanosomes, based on 18S rDNA partial sequences (~730 bp). Evolutionary relationships were determined by Maximum Likelihood, based on the Tamura-Nei model (Tamura et al., 2013). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site. Trypanosome species from bats are shown with an asterisk.

ing fox, using morphological and molecular techniques. This is the fourth trypanosome species to be reported in indigenous Australian bats and the first one associated with clinical disease. Unfortunately no genetic data was available for two of the previously reported bat-derived trypanosomes in Australia (T. pteropi and T. hipposideri). Trypanosoma pteropi was described as having a slender body (total length 18–22 μm; width 2–4 μm), an underdeveloped undulating membrane and a long free flagellum whereas T. hipposideri is very small and slender (total length 10.5–13 μm; width 1.5–2 μm), with a delicate short free flagellum at the anterior end (Breinl, 1913; Mackerras, 1959). Statistical analysis however, revealed that T. teixeirae sp. n. was significantly larger than both T. pteropi and T. hipposideri, even though they had several other overlapping morphometric features. However, morphology alone is not a reliable tool to delimit trypanosome species due to the interspecific similarities and intraspecific variability (Dunn et al., 1963; Marinkelle, 1966; Dunn, 1968).

Besides the typical trypomastigotes, two round forms (sphaeromastigotes or round epimastigotes) were also observed. The term ‘sphaeromastigote’ (Brack, 1968) refers to the parasite body shape
Phylogenetic relationships of Trypanosoma teixeirae sp. n. with other trypanosomes, based on gGAPDH partial sequences (~775 bp). Evolutionary relationships were determined by Maximum Likelihood, based on the Tamura-Nei model (Tamura et al., 2013). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site.

Fig. 3. Phylogenetic relationships of Trypanosoma teixeirae sp. n. with other trypanosomes, based on gGAPDH partial sequences (~775 bp). Evolutionary relationships were determined by Maximum Likelihood, based on the Tamura-Nei model (Tamura et al., 2013). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site. Trypanosome species from bats are shown with an asterisk.

only and has been applied without reference to the flagellar development. However, as these forms may occur within different stages of the parasite’s development, it is more appropriate to characterise the round organisms observed in the present study as round epimastigotes, considering both their body form and flagellar features (Elliott et al., 1974). This stage normally occurs in the interior of the cell, in vessels or in the insect gut.

Evolutionary reconstructions at both 18S rDNA and gGAPDH loci revealed that T. teixeirae sp. n. was genetically distinct from all known trypanosomes. The use of these two genes is recommended for taxonomic analysis of trypanosomatids and validation of new trypanosome species (Hamilton et al., 2004; Viola et al., 2009; Teixeira et al., 2011; Lima et al., 2012, 2013; Borghesan et al., 2013).

Phylogenetic analyses at both 18S rDNA and gGAPDH loci revealed that T. teixeirae sp. n. clustered within the T. cruzi clade together with all other bat-derived trypanosome species described to date, except T. livingstonei (which was positioned basal to the T. cruzi clade), T. evansi (which belongs to the T. brucei clade) and T. vegrandsis (which forms a separate group associated with other marsupial-derived trypanosomes found in Australia) (Hamilton et al., 2007; Botero et al., 2013; Lima et al., 2013; Austen et al., 2015; Carnes et al., 2015). At the gGAPDH locus, T. teixeirae sp. n. was closest to T. minasense and T. rangeli and exhibited 14% and 15% genetic
distance from these two species respectively. T. minasense has been found in neotropical non-human primates from South America (Zuccardi and Lourencco-de-Oliveira, 1998) whilst T. rangeli has been reported in a range of mammalian hosts including Brazilian bats (Maia da Silva et al., 2009). Although T. teixeirae sp. n. exhibited a relatively low (1%) genetic distance from its closest related species at the 18S rRNA locus, a similar pattern was observed when comparing other previously described species among each other. For instance, genetic distances between T. minasense and T. vespertilionis were 1% and 12% at the 18S rRNA and gGAPDH loci respectively. Trypanosomes have few morphological features detectable using light microscopy which can adequately delimit species (Gibson, 2009). Previous studies have reported that a genetic distance of 3.75% at the GAPDH gene is sufficient to delimit a new trypanosome species (Mclnnes et al., 2011). By this criterion, T. teixeirae sp. n. is clearly a separate species.  

Bat trypanosomes have been implicated in the evolutionary origin of T. cruzi, the causative agent of Chagas disease, one of the most important public health issues in South America (Hamilton et al., 2012; Bonney, 2014). The ‘bat-seeding’ theory suggests that T. cruzi evolved from within a broad clade of bat-derived species, which have made the switch into terrestrial mammals (Hamilton et al., 2012; Lima et al., 2013). The theory also implies that these arboreal trypanosomes species could potentially be evolutionary precursors for the terrestrial trypanosome lineage within Australian mammals (Hamilton et al., 2012; Lima et al., 2013; Thompson et al., 2014).  

It is therefore possible that T. teixeirae sp. n. could be the precursor of three marsupial-derived trypanosomes belonging to the T. cruzi clade: T. sp. H25 (Averis et al., 2009), T. sp. AP-2011-64 (Paparini et al., 2011) and T. sp. AB–2013–G8 (Botero et al., 2013). As most native bat species remain unsampled (Thompson et al., 2014), future studies are required to provide more evidence to support the ‘bat-seeding’ theory in Australia and elucidate evolutionary relationships between trypanosomes.  

Similarly to most bat trypanosomes described worldwide, the prevalence, distribution, vectors, life cycle and zoonotic potential of T. teixeirae sp. n. remain unclear. Therefore, more studies comprising a larger sample size are required to better understand the prevalence and clinical impacts of T. teixeirae sp. n. on bat populations, taking into account ecological and stress factors that could play a role in the expression of clinical disease.

References


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Note: due to copyright restrictions, the appendix above can be found at:

Haematozoa of wild catfishes in northern Australia

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1. Introduction

Parasitic haematozoa have been reported in a wide range of fish species worldwide. The most frequently reported haematozoa of fishes are kinetoplastids of the genera Trypanosoma and Trypanoplasma (Cryptobia), and apicomplexans belonging to genera of haemogregarines (Woo, 2006). Trypanosomas and haemogregarines are heteroxenous, and are believed to be transmitted to host species either through ingestion of an intermediate host, or via the bite of an infected tick or other vector. Several studies in Australia, for example, suggest that haematozoa such as trypanosomes may be contributing to the decline of endangered terrestrial mammal species such as the wombat Bettongia penicillata (Botero et al., 2013; Thompson et al., 2014), and Gilbert’s potoroo Potorous gilbertii (Austen et al., 2009) that are already threatened by wider ecosystem changes. Like many of Australia’s mammals, native freshwater fishes in Australia are highly endemic, and are increasingly

Only two species of haematozoa have been recorded from Australian freshwater teleosts. Johnston and Cleland (1910) recorded Trypanosoma bancrofti in freshwater catfish Tandanus tandanus in Queensland, and Trypanosoma anguilllicola in Australian marbled eel Anguilla reinhardti from New South Wales and Queensland. Mackerras and Mackerras (1961) recorded T. bancrofti and T. anguilllicola from the same host species. Although systematic parasite surveys of native fish species are increasing in Australia, most do not involve examination of blood samples, and therefore it is likely many haematozoan species have not been recorded (Adlard and O’Donoghue, 1998).

The effects of haematozoan infections on individual Australian freshwater fish or the health of fish populations are unknown. Parasites may influence host population dynamics by directly affecting host morbidity and mortality, modulating host growth and reproduction, and altering the likelihood of predation in the wild (Barber et al., 2000). Several studies in Australia, for example, suggest that haematozoa such as trypanosomes may be contributing to the decline of endangered terrestrial mammal species such as the wombat

Received 26 October 2017; Received in revised form 8 December 2017; Accepted 13 December 2017
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threatened by anthropogenic habitat alteration, including the introduction of invasive alien species, exotic disease emergence, and habitat destruction. As certain haemoparasite species are pathogenic to fishes (Ferguson and Roberts, 1975; Khan, 1985; MacLean and Davies, 1990; Woo, 2006), they represent a potential threat to wild populations already under pressure.

Ariid andplotoid catfishes represent a large component of total fish biomass in many northern Australian rivers (Bishop et al., 2001; Jardine et al., 2012), and silver cobbler Neoarius midgleyorum forms the basis of Western Australia’s only freshwater finfish fishery. Eel-tailed catfish *Tandanus tandanus* is currently listed as Threatened in Victoria under the Flora and Fauna Guarantee Act 1988 (Department of Sustainability and Environment Victoria, 2005), and as Endangered in the Murray-Darling Basin in New South Wales (NSW) under the NSW Fisheries Management Act 1994 (Fisheries Scientific Committee, 2008). Freshwater catfishes are often host to a highly diverse range of tissue parasites (Lymberty et al., 2010), however, no widespread study on the hematooza of catfishes has been undertaken in Australia. Here, we report on the haematooza of catfishes sampled from freshwater systems in northern Australia, and investigate the effect of fish size and species on parasite prevalence.

2. Materials and methods

2.1. Sample collection and preparation

Native catfishes were sampled from 11 localities across Western Australia, Queensland and the Northern Territory, using a combination of fyke nets, handlines and electrofishing, between May 2014 and February 2015 (Table 1; Fig. 1). Fishes were euthanised using a prolonged anaesthetic bath of isoeugenol (Aquat, Lower Hutt, New Zealand), examined by eye for ectoparasites, and body weight and total body length were recorded. Blood was collected by caudal vertebral venepuncture, or following excision of the caudal peduncle in small fish, elongated anaesthetic bath of isoeugenol (AquiS, Lower Hutt, New Zealand), killed by decapitation, and stained with Wright-Giemsa (Kinetic, Caboolture, Queensland).

2.2. Microscopic evaluation

Blood smears were systematically scanned using 10x objective lens, followed by closer examination with 40x (high-dry) and 100x oil immersion. A combination of traits separating the groups. All statistical analyses were implemented in JMP®, Version 10.0 (SAS Institute Inc., Cary, NC).

3. Results and discussion

Blood smears from 189 catfishes, representing six species, were examined (Table 1). No haematooza were observed in fishes from the considered uninfected if no haematooza were observed after 15 min of scanning with the 100x oil immersion objective lens (Salkeld and Schwarzkopf, 2005). Histological sections of tissues from infected fishes were examined for the presence of tissue and blood borne parasite stages. Slides were examined on an Olympus BX41 laboratory microscope, and images taken on an Olympus BX51 system microscope, using an Olympus DP70 microscope digital camera and software (www.olympus.com).

2.3. Infection parameters

Prevalence was estimated separately for haemogregarine and trypansom parasities (see Results), for each fish species in each locality, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000). Fisher exact tests were used to compare differences in prevalence between fish species or genera. Differences in length and weight between infected and uninfected fishes were tested using a non-parametric Wilcoxon test, with a normal approximation.

2.4. Morphometric analyses

Digital images were used to measure key morphological features of haemogregarines (Table 2), and trypansomes (Table 3), as utilized by Smit et al. (2006) and Mackerras and Mackerras (1961) respectively, using Image J software (open source Java image processing program, available from http://imagej.net/Downloads; Schindelin et al., 2012). Trypanosomes were divided into two different morphological groups on the basis of one morphological trait (KN; see Results). Differences between these groups over all other morphological traits were tested using multivariate analysis of variance (MANOVA) and differences between groups for each trait were tested using one-way analyses of variance (ANOVA), with a Bonferroni correction to maintain an experiment-wide error rate of 0.05. All morphological data were log-transformed and the residuals from all analyses were normally distributed. Where the MANOVA showed a significant difference between groups, stepwise discriminant analysis was used to find the best combination of traits separating the groups. All statistical analyses were implemented in JMP®, Version 10.0 (SAS Institute Inc., Cary, NC).

### Table 1

Haematooza present in catfishes by species and collection location.

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>Latitude (+ S)</th>
<th>Longitude (+ E)</th>
<th>Fish species collected (n)</th>
<th>Prevalence (95% CI)</th>
<th>Trypanosomes</th>
<th>Haemogregarines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brisbane River</td>
<td>27°5447</td>
<td>152°7837</td>
<td><em>Neoarius graeffi</em> (20)</td>
<td>0 (0.050–0.576)</td>
<td>0</td>
<td>0.350 (0.167–0.576)</td>
</tr>
<tr>
<td>Burnett River</td>
<td>25°2304</td>
<td>152°0116</td>
<td><em>Neoarius graeffi</em> (16)</td>
<td>0.062 (0.003–0.305)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Barron River</td>
<td>17°2611</td>
<td>145°5378</td>
<td><em>Tandanus tandanus</em> (18)</td>
<td>0.111 (0.020–0.330)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bloomfield River</td>
<td>15°9868</td>
<td>145°2882</td>
<td><em>Tandanus tropicanus</em> (19)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tully Catchment</td>
<td>17°8818</td>
<td>145°8412</td>
<td><em>Tandanus tropicanus</em> (18)</td>
<td>0.333 (0.156–0.586)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Palm Tree Creek</td>
<td>21°1540</td>
<td>148°7266</td>
<td><em>T. tandanus</em> (3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mary River (site A)</td>
<td>26°0342</td>
<td>152°5106</td>
<td><em>Neoarius graeffi</em> (10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mary River (site B)</td>
<td>26°3319</td>
<td>152°7020</td>
<td><em>Nessilurus hyrtli</em> (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Goondaloo Creek</td>
<td>19°3232</td>
<td>146°7630</td>
<td><em>Neoarius graeffi</em> (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ord River</td>
<td>15°7932</td>
<td>128°7177</td>
<td><em>Nessilurus hyrtli</em> (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rapid Creek</td>
<td>12°3955</td>
<td>130°8722</td>
<td><em>Neoarius midgleyorum</em> (13)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* At these sites, a number of locations were used to capture the required number of fishes and the coordinates refer to the modal locality.
Northern Territory (n = 29) or Western Australia (n = 24). Haematozoa were observed in 19.1% (n = 26, 95% CI = 13.39–26.54%) of fish sampled in Queensland, and infected fish were present in 62.5% (n = 5, 95% CI = 30.6–86.3%) of rivers sampled in Queensland. Specifically, haematozoa were detected in the blood of 32.4% (n = 12, 95% CI = 19.6–48.5%) of T. tandanus, 17% (n = 8, 95% CI = 8.9–30.1%) of N. graeffei and 16.2% (n = 6, 95% CI = 7.7–31.1%) of T. tropicanus sampled in Queensland, and were not observed in any sampled N. ater (n = 13) or N. hyrtlii (n = 2). All catfishes appeared healthy on external examination, and no evidence of ectoparasites were observed.

Monomorphic intraerythrocytic inclusions consistent with haemogregarine parasites (Davies, 1995) were observed in 35% (n = 7, 95% CI = 18.1–56.7%) of Neoarius graeffei sampled from the Brisbane River (Fig. 2). Haemogregarines were slender in shape with slightly tapering ends (Table 2), light blue cytoplasm and a central nucleus containing 9–13 coarse chromatin clumps on Wright-Giemsa stained smears.

Table 2
Dimensions and standard errors (S.E.) of morphological features of haemogregarines observed in N. graeffei sampled from the Brisbane River.

<table>
<thead>
<tr>
<th>Feature</th>
<th>No. of organisms measured</th>
<th>Observed range (μm)</th>
<th>Mean ± S.E. (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>43</td>
<td>10.418–14.191</td>
<td>12.1933 ± 0.13157</td>
</tr>
<tr>
<td>W</td>
<td>43</td>
<td>1.682–3.213</td>
<td>2.2915 ± 0.04851</td>
</tr>
<tr>
<td>NL</td>
<td>35</td>
<td>2.915–5.052</td>
<td>4.0336 ± 0.105037</td>
</tr>
<tr>
<td>NW</td>
<td>34</td>
<td>0.997–2.03</td>
<td>1.6225 ± 0.046642</td>
</tr>
</tbody>
</table>

* TL = total length (measured along midline), W = width (measured across nucleus), NL = length of nucleus, NW = width of nucleus.

Northern Territoy (n = 29) or Western Australia (n = 24). Haematozoa were observed in 19.1% (n = 26, 95% CI = 13.39–26.54%) of fishes sampled in Queensland, and infected fishes were present in 62.5% (n = 5, 95% CI = 30.6–86.3%) of rivers sampled in Queensland. Specifically, haematozoa were detected in the blood of 32.4% (n = 12, 95% CI = 19.6–48.5%) of T. tandanus, 17% (n = 8, 95% CI = 8.9–30.1%) of N. graeffei and 16.2% (n = 6, 95% CI = 7.7–31.1%) of T. tropicanus sampled in Queensland, and were not observed in any sampled N. ater (n = 13) or N. hyrtlii (n = 2). All catfishes appeared healthy on external examination, and no evidence of ectoparasites were observed.

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Rarely, free parasites were observed attached to an erythrocyte remnant. No haemogregarine species has previously been recorded from Neoarius graeffei. The majority of haemogregarine species are reported from marine fishes (Davies, 1995), and many reports of haemogregarines from freshwater fishes are from euryhaline species that may have spent time in the marine environment (Davies, 1995). Several haemogregarines from marine fishes exhibit a wide host-range (Davies, 1995) and, as some populations of N. graeffei are euryhaline, there exists the possibility of transmission from inshore marine fishes. Superficial morphological similarities exist between the haemogregarines observed in this study and intraerythrocytic Haemogregarina balistapi n. sp. described in orange-lined triggerfish Balistapus undulatus from Lizard Island, QLD (Smit et al., 2006), however further studies are required for

Table 3
Means and standard errors (S.E.) of morphological features of the broad and slender trypanosomes observed in this study.

<table>
<thead>
<tr>
<th>Trypanosome forms</th>
<th>Morphological feature</th>
<th>n</th>
<th>TL ± S.E.</th>
<th>W ± S.E.</th>
<th>PK ± S.E.</th>
<th>KN ± S.E.</th>
<th>NA ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad</td>
<td></td>
<td>29</td>
<td>56.6 ± 1.40</td>
<td>5.47 ± 0.287</td>
<td>1.69 ± 0.230</td>
<td>25.2 ± 0.613</td>
<td>25.16 ± 0.716</td>
</tr>
<tr>
<td>Slender</td>
<td></td>
<td>32</td>
<td>22.4 ± 0.396</td>
<td>1.96 ± 0.0926</td>
<td>0.724 ± 0.0868</td>
<td>9.86 ± 0.341</td>
<td>9.11 ± 0.264</td>
</tr>
</tbody>
</table>

* TL = total length (measured along midline), W = width (measured across nucleus, includes undulating membrane), PK = posterior to kinetoplast (distance between the kinetoplast, and the posterior end of the trypomastigote body), KN = kinetoplast to nucleus (distance between the kinetoplast, and the posterior edge of the nucleus), NA = nucleus to anterior (distance between anterior edge of the nucleus, to the anterior end of the trypomastigote body).
molecular identification.

Trypomastigotes of Trypanosoma spp. were observed in 32.4% (n = 12, 95% CI = 19.6–48.5%) of Tandanus tandanus, 16.2% (n = 6, 95% CI = 7.65–31.1%) of Tandanus tropicanus, and 2.1% (n = 1, 95% CI = 0.380–11.1%) of Neoarius graeiei sampled in Queensland (Table 1). Over all rivers in which trypanosome infections were found, Tandanus spp. were more likely to be infected than Neoarius spp. or Neosilurus spp. (Fisher exact test, p = .001). However, fish species is confounded with site in these analyses. At both sites where Tandanus spp. were sampled with co-existing Neoarius spp. or Neosilurus spp. (Mary River, sites A and B), trypanosomes were present only within Tandanus spp., at prevalence rates of 0.250 (n = 2) at the first site and 1 (n = 8) at the second site. Mary River site A was the only site in which sufficient numbers of different species were captured for analysis; although Tandanus tandanus was more heavily infected than Neoarius graeiei, the difference in prevalence was not significant (Fisher exact test, p = .18). The absence of trypanosomes in Neosilurus and Neoarius species co-existing with infected Tandanus species suggests, however, that the trypanosomes may be host specialists.

The absence of haematozoa from fishes collected in the Northern Territory and Western Australia may be influenced by the fact that larger numbers of catfishes were sampled from Queensland (n = 136) than the Northern Territory (n = 29) and Western Australia (n = 24), and that Tandanus species, clearly a species susceptible to trypanosome infection, are not present in these localities. However, it may also reflect the absence of suitable vectors or transmission dynamics (i.e. host/vector interaction) for haematozoa in these locations. Aquatic leeches and isopod crustaceans may serve as vectors for trypanosomes and haemogregarines in aquatic species (Hamilton et al., 2005; Smit et al., 2006; Woo, 2006; Curtis et al., 2013). Within Australia, Trypanosoma binneyi, which falls into a clade of trypanosomes from aquatic hosts, is believed to be transmitted to platypuses Ornithorhynchus anatinus by aquatic leeches (Paparini et al., 2014), however similar studies have not been conducted for wild freshwater fishes in Australia, and no ecto-parasites were observed in this study to suggest a potential vector.

The absence of haematozoa from N. ater and N. hyrtlii sampled in Queensland may be influenced by the fact that few Neosilurus species were sampled (n = 15), compared to Tandanus (n = 74) or Neoarius species (n = 47). However, it may also be due to differences in habitat preference or behaviour, which may influence exposure to suitable vectors. Neosilurus ater and Neosilurus hyrtlii are nocturnal, shoaling species (Lintermans, 2007; Burrows and Perna, 2006), frequently recorded in slow or still waters with a sandy or muddy substratum (Pusey et al., 2004a). N. graeiei occur in a wide range of habitats (Kailola and Pierce, 1988; Pusey et al., 2004b) across a range of salinities, and may undertake lateral movement into floodplains (Pusey et al., 2004b). T. tropicanus is a newly described species, however it is closely related to T. tandanus, which is a largely nocturnal species with a limited home range and high site fidelity (Koster et al., 2015), that is most frequently reported in lentic habitats (Allen et al., 2002; Lintermans, 2007) where macrophytes and wood provide cover (Koster et al., 2015).

For both species of Tandanus, infected fishes were larger than uninfected fishes, possibly due to the greater potential for parasite exposure over time, and a larger surface area of larger fishes for the attachment of vectors. For T. tandanus, the mean length of infected fish was 39.46 cm (SE 2.52 cm) and the mean weight was 631.25 (97.25) g compared to 31.91 (1.85) cm and 426.59 (71.82) g for uninfected fish (z = 2.16, P = .03 for length; z = 1.80, P = .07 for weight). For T. tropicanus, the mean length of infected fish was 19.13 (2.28) cm and the mean weight was 19.42 (11.84) g for uninfected fish (z = 1.92, P = .05 for length and z = 1.88, P = .06 for weight).

Two morphological forms of trypanosomes were observed in blood smears, and identified as “broad” and “slender” trypomastigotes. Both forms were elongate, with tapering posterior and anterior ends, a bacilliform cytoplasm on Wright-Giemsa-stained slides, a terminal kinetoplast, and no apparent free flagellum. Broad trypomastigotes contained a prominent, lightly eosinophilic nucleus on Giemsa-stained slides, and a well-developed undulating membrane (Fig. 3), however these structures were less prominent in slender trypomastigotes (Fig. 4). Broad and slender trypomastigotes could be differentiated on the basis of non-overlapping measurements in the distance between the kinetoplast and nucleus (KN < 15 μm for slender forms and KN > 20 μm for broad forms). From the MANOVA, there was a significant difference between groups over all other morphological traits (Wilks λ = 0.05; F4,56 = 246.22, P < .0001) and univariate tests also found significant differences between groups for total length (TL; F1,59 = 978.38, width (W; F1,59 = 210.88, distance between posterior end and kinetoplast (PK; F1,59 = 12.32) and distance between nucleus and anterior end (NA; F1,59 = 580.92) (P < .05 for all tests, with the Bonferroni) (Table 3). Discriminant analysis correctly classified 100% of cases along one canonical discriminant function, loading most heavily for TL (0.93), followed by PK (−0.22), W (0.15) and NA (0.07).
Comparison of the morphological features of broad and slender form trypomastigotes observed in this study, with Trypanosoma bancrofti.

<table>
<thead>
<tr>
<th>Trypanosome</th>
<th>Morphological features*</th>
<th>TL</th>
<th>W</th>
<th>PK</th>
<th>KN</th>
<th>NA</th>
<th>FF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad</td>
<td>41.3–71.0</td>
<td>3.43–9.01</td>
<td>0–4.55</td>
<td>20.5–30.8</td>
<td>15.7–33.7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Slender</td>
<td>19.1–30.3</td>
<td>1.04–3.07</td>
<td>0–1.89</td>
<td>7.18–14.8</td>
<td>5.86–12.9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>T. bancrofti (“narrow form”)a</td>
<td>27–31</td>
<td>&lt; 2.3</td>
<td>1</td>
<td>8–12.5</td>
<td>9</td>
<td>4–11</td>
<td></td>
</tr>
<tr>
<td>T. bancrofti (“broad form”)a</td>
<td>50–53.5</td>
<td>4.45–7</td>
<td>1.78</td>
<td>12.5</td>
<td>11</td>
<td>21.5</td>
<td></td>
</tr>
<tr>
<td>T. bancrofti (“intermediate form”)b</td>
<td>34</td>
<td>3.5</td>
<td>0</td>
<td>10.5–11.5</td>
<td>7.5–10</td>
<td>9–11.5</td>
<td></td>
</tr>
<tr>
<td>T. bancrofti</td>
<td>21.5–23</td>
<td>2.25</td>
<td>0</td>
<td>10.5–11.5</td>
<td>7.5–10</td>
<td>9–11.5</td>
<td></td>
</tr>
</tbody>
</table>

* TL = total length (measured along midline), W = width (measured across nucleus, includes undulating membrane), PK = posterior to kinetoplast (distance between the kinetoplast, and the posterior end of the trypanomastigote body), KN = kinetoplast to nucleus (distance between the kinetoplast, and the posterior edge of the nucleus), NA = nucleus to anterior (distance between anterior edge of the nucleus, to the anterior end of the trypanomastigote body), FF = free flagellum.
a Johnston and Cleland (1910).
b Mackerras and Mackerras (1960).

Broad trypomastigotes were found in T. tropicanus from the Tully River (Fig. 3), while slender trypomastigotes were present in T. tandanus from the Barron and Mary River Site A, and N. graeffei from the Burnett River (Fig. 4). Both broad and slender trypomastigotes were observed concomitantly within individual T. tandanus from the Mary River Site B (Fig. 5). The ranges of all morphological features for slender form trypomastigotes observed in this study overlap with T. bancrofti reported by Mackerras and Mackerras (1961), and the “narrow form” T. bancrofti reported by Johnston and Cleland (1910) (Table 4). The length, width and PK ranges for broad form trypomastigotes in this study, and “broad form” T. bancrofti (Johnston and Cleland, 1910) also overlap (Table 4). T. bancrofti is reported to be highly polymorphic (Johnston and Cleland, 1910; Mackerras and Mackerras, 1961), however this has not been confirmed with molecular methods. It is possible that the two forms of trypomastigotes in this study are morphological variants of one polymorphic species, as has been previously suggested for T. bancrofti (Johnston and Cleland, 1910; Mackerras and Mackerras, 1961), however it is also possible that they may be different species, particularly considering their concomitant occurrence in individual fish hosts. Further molecular studies are required to test these hypotheses.

No tissue stages or pathology associated with haematozoan infection was observed on examination of histological sections, and the parasitism was observed on examination of histological sections, and the parasites themselves are important contributors to biodiversity. Australian freshwater ecosystems are increasingly threatened by anthropogenic practices, and identification and knowledge of the potential threats to wild fish health, such as haematozoa, is important for the conservation of Australia’s unique freshwater biodiversity.

Acknowledgements

Catfishes in this study were collected as part of the Fisheries Research and Development Corporation (FRDC) project 2012-050 “Edwardsiella ictaluri survey in wild catfish populations,” which is supported by funding from the Fisheries Research and Development Corporation on behalf of the Australian Government. This work was also supported by an Australian Government Research Training Program Scholarship. All fishes were collected under Animal Ethics Permit RW2618/13 approved by the Murdoch University Research Ethics committee. Thanks to the many people who assisted with collection and processing of samples: G. Dally and M. Hammer (Museum and Art Gallery of the Northern Territory), B. Hanekom, D.L. Morgan and J. Keleher (Murdoch University), B.C. Ebner, J. Donaldson and A.M. Davis (James Cook University), and CSIRO Land and Water, L. Foyle (James Cook University), S. Brooks (Queensland Department of Agriculture and Fisheries).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2017.12.002.

References


