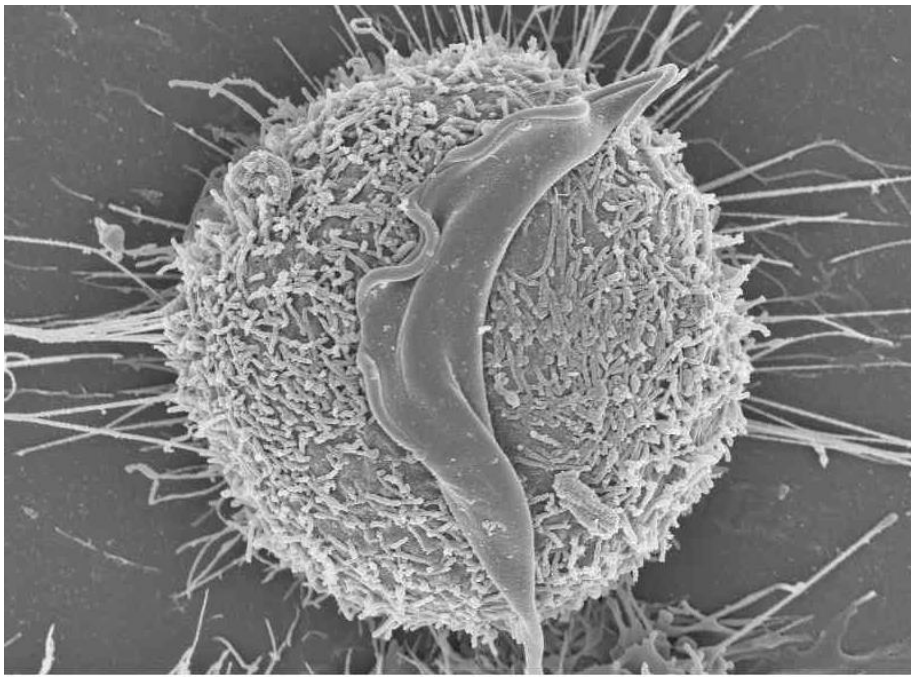
Genotypic and phenotypic diversity of trypanosomes infecting Australian marsupials and their association with the population decline of the brush-tailed bettong or woylie (*Bettongia penicillata*)



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This thesis is presented for the degree of Doctor of Philosophy at

Murdoch University, 2014

Declaration

I certify that this full thesis is the own account of my research, and it does not contain any material previously published or submitted for a degree at any other tertiary educational institution.

Luz Adriana Botero Gomez

Preface

The research described herein was conducted at Murdoch University and at the Centre for Microscopy, Characterisation and Analysis at the University of Western Australia (CMCA). This thesis is written in a "thesis by publication" format and is composed of published and about to be submitted manuscripts. Chapters 2 and 3 are part of a published paper in the *International Journal for Parasitology: Parasites and Wildlife* 2013 (attached in Appendix I). The addition of co-authors in this paper was to acknowledge the contributions of collaborators who provided or helped in the collection of samples, helped with the analysis of the data, as well as provided valuable editorial comments.

Chapter 4, 5, 6 and 7 are written in a scientific manuscript format and are in the process of submission for publication as separate papers in different scientific journals. Co-authors are included to acknowledge their contributions with the analysis of the data and with constructive editorial comments.

This dissertation is an original intellectual product of the author. The author carried out all the development and writing.

Acknowledgements

These four years have been a challenging trip, with both ups and downs, but fortunately, I have been surrounded by many good friends, family and excelent supervisors - this work was much easier to complete with all their help and support!

Firstly, special thanks to my principal supervisor professor Andy Thompson, who has shared his knowledge and time with me. I am very grateful to him for giving me the opportunity and freedom to determine the direction of my research, along the lines that we both thought were best. Thank you for all the words of encouragement and motivation when most needed.

I would like to thank my other supervisor, associate professor Christopher Peacock, for all his helpful comments and suggestions on my work and his continual support. Huge thanks to Associate professor Peta Clode for all her endless support and for sharing with me all her invaluable expertise in microscopy techniques, without her help we wouldn't have all the beautiful images that this thesis contains.

I would like to thank also Dr Adrian Wayne from the Department of Parks and Wildlife (DPaW) for his usefull comments and suggestions. Thanks to the facilities of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis in the University of Western Australia. Thanks to the Western Australian Government's State NRM Program and to DPaW for providing funding for this project.

Special thanks go to all my colleagues and friends in the University who have worked with me over the years. Thanks to Crystal Cooper, Sarah Keatley for their help and many useful discussions, comments and suggestions. Special thanks to Louise Pallant for her continuous help, friendly advices, and words of encouragement when most needed. Special thanks also to my colleague and friend Craig Thompson - thanks for all the nice chats and help with all the field work. I would like to thank also my friend Devinia Au, she especially deserves heartfelt thanks for getting into every single chapter of my thesis in such a short period of time, and for all her editing comments.

Thanks and love to my mother Ofelia for her encouragement and support. Undoubtedly, my husband Juandi deserves a huge special word of thankfulness for his moral support, for his patience and love; he had dealt with all the frustrations and without his support this work would not have been done - thank you very much Corazon!

Abstract

Trypanosomes are flagellated blood parasites that are capable of infecting virtually all classes of vertebrates. They range from non-pathogenic species to those that are highly pathogenic and are the causative agents of many diseases of medical and veterinary importance. While much is known of their impact on human health or economic development, a great deal less is known of those associated with wildlife.

Within Australia, trypanosomes have been found naturally infecting a wide range of native marsupials, most of which are considered threatened or endangered. However, their research has largely been confined to the description of trypanosome morphology in blood, and a complete lack of information regarding their life cycle, virulence, and pathogenicity is evident. This study therefore, aimed to investigate the genotypic and phenotypic diversity of *Trypanosoma* spp. infecting Western Australia marsupials and to determine their potential pathogenicity with particular emphasis in the critically endangered marsupial, the woylie (*Bettongia penicillata*). The genotypic characterisation was achieved using a combination of sequencing and phylogenetic analysis of trypanosomes in the blood and tissues of nine different marsupial species, as well as the sequencing of partial fragments of the minicircles of the kinetoplast DNA of trypanosomes isolated in culture. The phenotypic characterisation involved a combination of histology, microscopy techniques, and *in vitro* experiments of cell infection and drug susceptibility.

Results revealed that eight different genotypes belonging to three different *Trypanosoma* species: *T. copemani*, *T. vegrandis*, and *T.* sp H25 were found infecting

woylies (*Bettongia penicillata*), quendas (*Isoodon obesulus*), quokkas (*Setonix brachyurus*), tammar wallabies (*Macropus eugenii*), banded hare wallabies (*Lagostrophus fasciatus*), boodies (*Bettongia lesueur*), Chuditches (*Dasyurus geoffroii*), common brush tailed possums (*Trichosurus vulpecula*), and western grey kangaroos (*Macropus fuliginosus*). However, the woylie was the only marsupial species where single individuals and single tissues were co-infected with genotypes belonging to the three different *Trypanosoma* species. Furthermore, *T. copemani* G2, the predominant trypanosome in the declining population of woylies, was shown to be able to infect tissue cells and generate a strong immune response characterised by tissue degeneration and necrosis in vital organs, suggesting an association between these infections and the decline of the woylie. Comparative analysis between *T. copemani* G2 and the pathogenic *T. cruzi* showed not only similarities in their capacity to infect tissue cells, but also in drug susceptibility and kinetoplast DNA organisation.

In summary, this study not only contributes valuable information towards directing management decisions for endangered species where trypanosomes are known to be present at high prevalence levels, but also provides new knowledge about the evolutionary biology and relationships that Australian trypanosomes have with the exotic and pathogenic *T. cruzi*.

Publications and Presentations

Part of the work presented in this thesis has been published and accepted for presentation in scientific conferences as described below:

Publications

Adriana Botero, Craig K. Thompson, Christopher S. Peacock, Peta L. Clode, Philip K. Nicholls, Adrian F. Wayne, Alan J. Lymbery, RC Andrew Thompson. Trypanosomes genetic diversity, polyparasitism and the population decline of the critically endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*). International Journal for Parasitology: Parasites and Wildlife, **2013**. 2: 77-89.

Thompson CK, **Botero A**, Wayne AF, Godfrey SS, Lymbery AJ, Thompson RA. Morphological polymorphism of *Trypanosoma copemani* and description of the genetically diverse *T. vegrandis* sp. nov. from the critically endangered Australian potoroid, the brush-tailed bettong (*Bettongia penicillata*) (Gray,1837). Parasites and Vectors **2013**, 6:121.

Presentations at conferences

Adriana Botero, Joseph Shlomai, Peta Clode, Andrew Thompson. 2014. Novel insights into the Kinetoplast DNA structure of the Australian *Trypanosoma copemani.* Canberra, Australia. The Australian Society for Parasitology annual conference.

Adriana Botero, Christopher Peacock, Peta Clode, Andrew Thompson. 2013. Infection kinetics and drug susceptibility of the Australian *Trypanosoma copemani*: a comparative *in vitro* study with the pathogenic *Trypanosoma cruzi*. Perth, Australia.

24th International Conference for the World Association for the Advancement of Veterinary Parasitology.

Adriana Botero, Christopher Peacock, Peta Clode, Andrew Thompson. 2012. *Trypanosoma* infections as a contributory factor in the population decline of the endangered Australian marsupial, *Bettongia penicillata ogilbyi*. Tasmania, Australia. The Australian Society for Parasitology annual conference.

Adriana Botero, Craig Thompson, Andrew Smith, Philip Nicholls, Christopher Peacock, Adrian Wayne, Andrew Thompson. 2012. Genetic diversity of trypanosomes in Western Australian marsupials and the potential of mixed infections as a contributory factor in the population decline of brush-tailed bettong (*Bettongia penicillata*). Lion, France. The conference on wildlife diseases held by the Wildlife Disease Association (WDA) and European Wildlife Disease Association (EWDA).

Adriana Botero, Christopher Peacock, Peta Clode, Andrew Thompson. 2011. Biological and genetic characterisation of trypanosomes in Western Australian marsupials. Cairns, Australia. The Australian Society for Parasitology annual conference.

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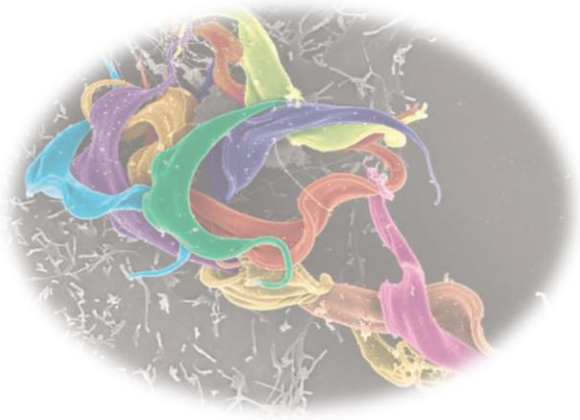
Abbreviations

KDNA	The kinetoplast DNA
Kb	Kilobases
Bp	Base pair
UMS	Universal Minicircle Sequence
UMSBP	Universal Minicircle Sequence Binding Protein
KFZ	Kineto-flagellar zone
RNAi	RNA interference
PBS	Phosphate buffered saline
DTT	Dithiothreitol
RPMI 1640	Roswell Park Memorial Institute 1640
HMI-9	Iscove's modified DMEM-based
MEM	Modified Eagles Medium
LIT	Liver Infusion Tryptose
BHI	Brain-Heart Infusion
FCS	Fetal Cow Serum
TEM	Transmission electron microscopy
SEM	Scanning electron microscopy
AX	Axoneme AX
PFR	Paraflagellar rod
PMA	Phorbol 12-myristate 13-acetate
CsCl	Cesium chloride
EtBr	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate

Chapter 1

General Introduction



1.1 Summary

Trypanosomes are protozoan parasites that are well known for their pathogenicity in humans and other mammals. However, while much is known about their life cycle and their impact on human health or economic development, a great deal less is known about those associated with wildlife. Within Australia, numerous *Trypanosoma* species and genotypes have been described naturally infecting a wide range of native marsupials. However, trypanosome research in Australian wildlife has largely been confined to studies on individual host species and their study has been limited to the description of their morphology in blood. This research project aimed to expand the knowledge of Australian trypanosomes by investigating their genetic diversity, biological behaviour in the marsupial host, and potential pathogenicity. Additionally, this project hoped to make a contribution towards understanding the relationships between *Trypanosoma* infections and the drastic decline of the endangered Australian marsupial, the woylie (*Bettongia penicillata*) (Wayne *et al*. 2013a). This review will, therefore, outline current knowledge of the diversity, geographic distribution, and host range of trypanosomes worldwide that are relevant to this research project. It will also summarise different aspects concerning life cycle, morphology, drug susceptibility, and pathogenicity of trypanosomes. Comparison with well-characterised *Trypanosoma* species might facilitate a better understanding of the different aspects of the biology of new and undescribed *Trypanosoma* species within Australian wildlife.

1.2 Trypanosomes and their hosts: a global summary

Trypanosomes are flagellate protozoan parasites that infect a wide range of vertebrates, invertebrates and plants (Simpson *et al*. 2006). They belong to the family

Trypanosomatidae, which also includes the genus *Leishmania*. Some of the most studied trypanosomes are a group of two human pathogens that are the cause of many deaths every year. Trypanosomes from the *Trypanosoma brucei* complex, *T. brucei gambiense* and *T. brucei rhodesiense*, are the agents of sleeping sickness that affects approximately 20,000 people, with 70 million being at risk of contracting the disease. In the new world, *Trypanosoma cruzi* is the causative agent of Chagas disease, which affects approximately 10 million people, with the highest incidence being in Latin America (WHO 2010). Both, *T. brucei* and *T. cruzi*, also infect wild animals, and can be transmitted from these to humans (zoonotic infections) (Miles *et al*. 2003). Wildlife are the natural reservoir hosts of trypanosomes, and are believed to "have learned to live in harmony" with the parasite (Legey *et al*. 1999). However, under certain circumstances such as stress caused by draught or starvation, and during concurrent infections, the innate resistance of wildlife to the infection with trypanosomes can be compromised, leading to an increase in parasitaemia, and severe pathological effects (Herrera *et al*. 2008, Mbaya *et al*. 2009). Some species from the *T. brucei* complex such as *T. brucei brucei, T. brucei congolense,* and *T. vivax,* are responsible for animal African trypanosomiasis or Nagana, which have an enormous impact on cattle health and production. Together with these trypanosomes from Africa that affect livestock, there are other species such as *T. equinum* and *T. equiperdum* that affect equines, *T. evansi* that affects dogs, wild mammals, cattle and other domestic animals, and *T. suis* that affects pigs (Ventura *et al*. 2002, Herrera *et al*. 2004).

Other species of trypanosomes have also been found to infect wildlife species across the world including birds, fish, amphibians, reptiles, rodents, baths, monotremes, primates, and marsupials (Hoare. 1972). Even though more than 100 species of avian

trypanosomes have been described to date (Baker. 1976, Bennett *et al*. 1992, Podlipaev. 1990), most of the descriptions from birds have been solely based on host specificity and did not provide molecular and/or morphological data for comparison (Sehgal *et al*. 2001, Zídková *et al*. 2012). So far, only *T. corvi*, *T. avium*, *T. culicavium*, *T. anguiformis*, *T. confusum* and *T. polygranularis* have been described based on morphology and phylogenetic data from birds from the families Corvidae, Accipitridae and Laniidae (Nandi *et al*. 1994, Sehgal *et al*. 2001, Valkiunas *et al*. 2011, Votýpka *et al*. 2012). The biological behaviour of avian trypanosomes in the vertebrate host is poorly understood. However, it is known that some species can be pathogenic to their host (Molyneux *et al*. 1983, Tarello. 2005).

Crocodilians, alligators, lizards, snakes and turtles inhabiting terrestrial and aquatic environments worldwide have long been known to be infected with trypanosomes (Hoare. 1972). Trypanosomes from amphibians and reptiles include *T. rotatorium* from frogs (Žičkus. 2002), *T. mega* from toads (Ashour *et al*. 1997), *T. grayi* and some uncharacterised species from crocodiles (Viola *et al*. 2009, Marcili *et al*. 2013), and *T. ralphi* and *T. terena* from alligators (Fermino *et al*. 2013).

Fish trypanosomes are believed to live extracellularly in the blood (Overath *et al*. 1998). However, in some animals, parasites may be detectable in the capillaries of internal organs such as kidney and heart (Lom *et al.* 1992). *T. carassii* has been found in the vascular system of a variety of cyprinid and some noncyprinid fishes (common carp) (Lom *et al*. 1992). *T. granulosum*, and *T. cobitis* have been described in the European eel (*Anguilla anguilla*), and in the stone loach (*Barbatula barbatula*) respectively (Letch. 1979, Davies *et al*. 2005). It has been shown that the control of the infection by

the immune system leads to a chronic state characterised by low or undetectable levels of parasites in the blood (Overath *et al*. 1999). However, some species has been shown to cause pathology; *Trypanosoma danilewskyi* has been shown to be pathogenic in juvenile common carp when inoculated with high doses of flagellates. Infection caused a severe anaemia and 50 % of mortality (Ahmed *et al*. 2011). Within Australia, an uncharacterised *Trypanosoma* sp. was found in the blood vessels, the choroid of the eye, the gill, and the dermis of moribund barramundis. Disease was characterised by intra-ocular haemorrhage and splenomegaly caused by these trypanosomes and it was suggested as the main cause of mortality (http://www.nt.gov.au/d/Content/File/p/Fish_Rep/FR098.pdf).

Rodent trypanosomes are considered non-pathogenic flea-borne parasites that are highly specific to their vertebrate hosts, where they live extracellularly in the blood (Albright *et al*. 1991). However, *T. lewisi*, a rodent trypanosome (Hoare. 1972) that has been found sporadically infecting humans (Sarataphan *et al*. 2007, Kaur *et al*. 2007, Verma *et al*. 2011, Shah *et al*. 2011), was suggested to be a likely cause of the extinction of *Rattus macleari* on Christmas Island in Australia in 1908 (D'Alessandro *et al*. 1991, Wyatt *et al*. 2008, MacPhee *et al*. 2013). This parasite has also been found extracellularly in kidney capillaries of infected rats (Ormerod et al. 1956). *T. grosi* and *T. evotomys* have been described infecting the wood mouse (*Apodemus sylvaticus*) (Hoare. 1972, Noyes *et al*. 2002), *T. microti* in the vasa recta of mouse kidneys (Monroy *et al*. 2000), and *T. lainsoni* in the spiny tree rat (*Mesomys hispidus*) (Naiff *et al*. 2013). The human pathogenic *T. cruzi* has also been found infecting Darwin's leaf-eared mouse (*Phyllotis darwini*), the Olive Grass Mouse (*Abrothrix olivaceus*), the

brush-tailed rat (*Octodon degus*), the long- tailed pygmy rice rat (*Oligoryzomis longicaudatus*), and Bennett's chinchilla rat (*Abrocoma benetti*) (Rozas *et al*. 2007).

Numerous species of bats have been reported to be infected with trypanosomes around the world. *T. vespertilionis* was reported in bats in Europe, the Americas, Africa and Asia; *T. dionisii* and *T. pipistrelli* in the Old World, particularly in Europe, and *T. cruzi marinkellei* in Colombia (Hoare. 1972, Marinkelle. 1976). *T. cruzi cruzi* (T.cBat) has been reported infecting the insectivorous bats *Noctilio albiventris* and *Myotis* spp. in Brazil (Marcili *et al*. 2009a), and the fruit-eating bat *Artibeus jamaicensis* in Panama (Pinto *et al*. 2012). *T. rangeli* was reported in the bats *Platyrrhinus lineatus* and *Artibeus planirostris* in Brazil (Maia da Silva *et al*. 2009). More recently, *T. erneyi* was described in Molossidae bats and *T. livingstonei* in the bats *Rhinolophus landeri* and *Hipposideros caffer* in Southeast Africa (Lima *et al*. 2012, Lima *et al*. 2013). Despite extensive knowledge about the occurrence of trypanosomes in bats around the world, little is known about their natural vectors and life cycle in both vertebrate and invertebrate hosts.

Characterised and uncharacterised species of trypanosomes have been described infecting non-human primates. Early studies have described *T. cyclops* from the Malaysian primates *Macaca nemestrina* and *Macaca ira,* and other uncharacterised *Trypanosoma* sp. in Asian primates (Weinman. 1972, Weinman *et al*. 1978). Primates captured in the Brazilian Amazon were found infected with *T. cruzi*, *T. minasense*, *T. devei* and *T. rangeli* (Ziccardi *et al*. 2000, Maia da Silva *et al*. 2008, Marcili *et al*. 2009b).

Marsupials account for the largest number of species of *Trypanosoma* described so far. Many species from the family Didelphidae, including *Didelphis marsupialis*, *Didelphis virginiana, Didelphis albiventris* (Rabinovich *et al*. 2001), and other marsupials such as *Philander frenata* (Legey *et al*. 2003), *Thylamys elegans* (Rozas *et al*. 2007), and *Dasypus novemcinctus*, are common hosts of *T. cruzi* (Bern *et al*. 2011). *T. peba* was described in the blood and smears of subcutaneous lymph nodes from armadillos (*Euphractus sexcinctus setosus*) in Brazil, and was shown to not affect tissues (Barrett *et al*. 1990), and *T. freitasi* has been described in the blood and anal glands of *Didelphis azarae* and *Didelphis marsupialis* (Hoare. 1972, Deane *et al*. 1990). *T. saloboense* was described in the blood of the Brazilian opossum *Monodelphis emiliae* (Lainson *et al*. 2008).

While the description of most of the wildlife trypanosomes is based on morphologically descriptions in blood and/or molecular analysis, a great deal is known about their biological behaviour in the vertebrate host, including their capacity to migrate to different tissues and to infect and replicate inside cells.

1.3 Australian trypanosomes and their hosts

Within Australia a number of native and exotic trypanosomes have been described from the blood of a broad range of native wildlife including birds, rodents, bats, monotremes, marsupials and fish, from which marsupials account for the highest number of species infected with trypanosomes (Thompson *et al*. 2014b).

Of the exotic trypanosomes established in Australia, *T. lewisi* was recorded in Maclear's Rat (*Rattus macleari*) and in the bulldog rat (*Rattus nativitatis*) (Mackerras. 1959,

Pickering, and Norris. 1996, Wyatt *et al*. 2008), and *T. nabiasi* in the introduced rabbit (*Oryctolagus cuniculus*) (Hamilton *et al*. 2005b). To date, 15 native species of *Trypanosoma* have been described from wildlife, including monotremes, birds, fish, bats, reptiles, amphibians and marsupials (Table 1). Numerous uncharacterised trypanosomes have also been described in the currawong (*Strepera sp*), Shark bay mouse (*Pseudomys fieldi*), bush rat (*Rattus fuscipes*), woylie (*Bettongia penicillata*), common wombat (*Vombatus ursinus*), eastern grey kangaroo (*Macropus giganteus*), swamp wallaby (*Wallabia bicolor*), brush tailed possum (*Trichosurus vulpecula*), chuditch (*Dasyurus geoffroii*), golden bandicoot (*Isoodon auratus*), southern brown bandicoot (*Isoodon obesulus*) and burrowing bettong (*Bettongia lesueur*) (Bettiol *et al*. 1998, Noyes *et al*. 1999, Hamilton *et al*. 2005b, Smith *et al*. 2008, Averis *et al*. 2009, Paparini *et al*. 2011). The fact that none of these species and genotypes has been described in any other part of the world other than Australia indicates they might be indigenous to Australia. Table 1 shows all the species of indigenous Australian trypanosomes and their respective hosts.

Table 1. Species of *Trypanosoma* naturally infecting native Australian wildlife

	Trypanosoma	**Host**	**References**
Monotremes	*T. binneyi*	Platypus (*Ornithorhynchus anatinus*)	(Mackerras. 1959, Noyes *et al*. 1999, Paparini *et al*. 2014)
Bats	*T. pteropi*	Black flying fox (*Pteropus alecto*)	(Johnston. 1916)
	T. hipposideri	Dusky roundleaf bat (*Hipposideros ater*)	(Mackerras. 1959)
Birds	*T. notophoyxis*	White-faced heron (*Ardea novaehollandiae*) Pacific reef heron (*Egretta sacra*)	(Breinl. 1913)
	T. eudyptulae	Little penguin (*Eudyptula minor*)	(Jones *et al*. 1989)
Reptiles	*T. chelodina*	Long-necked tortoise (*Chelodina longicollis*) Northern snapping turtle (*Elseya dentate*) Saw-shelled turtle (*Elseya latisternum*) Krefft's river turtle (*Emydura krefftii*) Murray turtle (*Emydura macquarii*)	(Johnston *et al*. 1909, Gray. 1830)
	T. phylluri	Southern leaf-tailed gecko (*Phyllurus platurus*)	(Mackerras. 1961)
	T. egerniae	Cunningham's skink (*Egernia cunninghami*) Tree skink (*Egernia striolata*)	(Mackerras. 1961)
Amphibians	*T. clelandi*	Ornate burrowing frog (*Limnodynastes ornatus*) Spotted grass frog (*Limnodynastes tasmaniensis*)	(Johnston, and Cleland. 1909)
Fish	*T. mackerasi*	Epaulette shark (*Hemiscyllium ocellatum*) Squatina tergocellatoides (*Ocellated catshark*)	(Lester *et al*. 1989)
Marsupials	*T. thylacis*	Southern brown bandicoot (*Isoodon obesulus*) Northern brown bandicoots (*Isoodon macrourus*)	(Mackerras. 1959)
	T. irwini	Koalas (*Phascolarctos cinereus*)	(McInnes *et al*. 2009)
	T. gilletti	Koalas (*Phascolarctos cinereus*)	(McInnes *et al*. 2011a)
	T. copemani	Quokkas (*Setonix brachyurus*) Gilbert's potoroo (*Potorous gilbertii*) Koalas (*Phascolarctos cinereus*)	(Austen *et al*. 2009, McInnes *et al*. 2011a)
	T. vegrandis	Woylie (*Bettongia penicillata*)	(Thompson *et al*. 2013)

1.4 Origin and taxonomy of trypanosomes

Trypanosomes belong to the family Trypanosomatidae (order Kinetoplastida) that includes also *Leishmania* parasites, *Crithidia fasciculata* (a parasite of insects) (Podlipaev *et al*. 2004), and free-living protozoa from the family Bodinidae such as *Bodo saltans* and *Bodo caudatus* (Podlipaev *et al*. 2010). They have been of major interest not only because of their importance as pathogens, but also because they have

diverged early from the eukaryotic lineage and developed distinct and well conserved subcellular structures such as the kinetoplast, a unique organelle that contains the mitochondrial DNA of the parasite (Hoare. 1972).

Different and conflicting theories about the evolutionary origin and phylogenetic relationships among trypanosomatids have been put forward over the last two decades (Stevens. 2014). Several phylogenetic studies have suggested trypanosomatids diverged from free-living bodonids (Simpson *et al*. 2002, Simpson *et al*. 2004a, Moreira *et al*. 2004, Deschamps *et al*. 2011). Hypotheses have emerged that aquatic bodonid protozoans adapted to parasitism in the intestinal tract of insects after being ingested accidentally, giving rise to insect trypanosomatids (Hamilton *et al*. 2004). Subsequently, trypanosomatids of hematophagus insects were transmitted to vertebrates, where they adapted to parasitism, giving rise to *Leishmania* and *Trypanosoma* parasites (Hoare. 1972). More recently, the discovery of a novel parasite of mosquitoes, *Paratrypanosoma confusum*, which branched in a phylogeny with very high support at the base of the family Trypanosomatidae - together with bodonids, could represent the missing link between the ancestral free-living bodonids and the derived parasitic trypanosomatids (Flegontov *et al*. 2013). *P. confusum* might provide new insights into our understanding of the evolution of parasitism within trypanosomatids.

The origin of all species within the genus *Trypanosoma* has also been controversial. The issue that seems to have offered most debate is that concerning the monophyly or paraphyly of trypanosomes (Simpson *et al*. 2006). Even with the advent of molecular methods, this conflict has not been resolved mainly due to the use of different gene sequences, number of taxa, outgroups and different phylogenetic methodologies in each

study (Maslov *et al*. 1996, Lukeš *et al*. 1997, Stevens *et al*. 1999b, Stevens *et al*. 2001, Hughes *et al*. 2003, Hamilton *et al*. 2004, Simpson *et al*. 2004b, Piontkivska *et al*. 2005). Molecular phylogenetic studies, based on comparisons of genes encoding mitochondrial and nuclear ribosomal RNAs (rRNA), showed trypanosomes are paraphyletic (Gomez *et al*. 1991, Maslov *et al*. 1995, Maslov *et al*. 1996). However, other studies that included more taxa, a broader range of host species, and protein-coding genes supported the monophyly of trypanosomes (Lukeš *et al*. 1997, Adjé *et al*. 1998, Wright *et al*. 1999, Hannaert *et al*. 1998, Hamilton *et al*. 2007, Leonard *et al*. 2011). The confusion over monophyly/paraphyly in the genus *Trypanosoma* indicates the need for caution when constructing and interpreting phylogenies, especially when the number of taxa used is low.

Despite the controversy regarding the phylogeny of trypanosomatids and the monophyly or paraphyly of the genus *Trypanosoma*, different clades have been defined with high phylogenetic support: the *T. brucei* clade, the *T. theileri* clade, the *T. lewisi* clade, the *T. cruzi* clade, the aquatic clade comprising the anuran clade and the fish clade, the crocodilian clade, the avian clade, and the lizard clade (Stevens *et al*. 1998, Stevens *et al*. 1999a, Hamilton *et al*. 2007, Fermino *et al*. 2013).

The *T. brucei* clade comprises exclusively mammalian trypanosomes of African origin. *T. brucei gambiense* and *T. b. rhodesiense* that affect humans, domestic animals and wildlife, and *T. b. brucei, T. b. congolense, T. vivax, T. equinum, T. equiperdum, T. suis,* and *T. evansi* that affect livestock and wildlife (WHO 2010). However, *T. evansi* and *T. vivax* were recently found in South America, and are believed to be the product of accidental introductions into the continent in domestic animals (Cortez *et al*. 2006).

The *T. theileri* clade contains trypanosomes from marsupials and placental mammals that are distributed worldwide from the tropics to near the Arctic Circle, with higher prevalence in tropical and neotropical areas. It includes *T. theileri* from buffalos, deer and cattle from America and Europe (Rodrigues *et al*. 2006), *T. cyclops* from Asian macaques (Weinman. 2012), and an uncharacterised trypanosome from an Australian wallaby *T. sp* ABF (Hamilton *et al*. 2005a).

The *T. lewisi* clade comprises trypanosomes from rodents. *T. lewisi* has also been found infecting monkeys, which is believed to be the product of host switching from their natural rodent species (Maia da Silva *et al*. 2010). *T. lewisi* and *T. lewisi*-like trypanosomes within this clade have been reported from America, Europe, Asia and Africa. This clade also includes *T. musculi*, *T. grosi*, and *T. microti*; all trypanosomes from mice (Maia da Silva *et al*. 2010).

The aquatic clade comprises mainly trypanosomes of aquatic vertebrates and amphibians, including an uncharacterised trypanosome from a turtle. Two small clades are present within this clade, the anuran and the fish clades (Hamilton *et al*. 2007). With a wide geographical distribution, the anuran clade comprises *T. rotatorium* and *T. mega* (Bardsley *et al*. 1972, Ashour, and Gaafar. 1997, Žičkus. 2002, Martin *et al*. 2009), and the fish clade comprises *T. carassii* (Lom *et al*. 1992), *T. granulosum*, and *T. cobitis* from Europe and Africa (Gibson *et al*. 2005).

The single robustly monophyletic avian clade comprises *T. avium*, *T. corvi*, *T. culicavium* and a high number of uncharacterised *Trypanosoma* species from birds

(Zídková *et al*. 2012). Trypanosomes within this clade occur in all continents except for the polar region (Baker. 1976, Apanius. 1991).

The *T. cruzi* clade includes species of the genus *Schizotrypanum* such as *T. cruzi*; trypanosomes of bats from the Old and New World, such as *T. cruzi marinkellei*, *T. dionisii*, *T. vespertilionis*, *T. conorhini*, and *T. erneyi*; it also includes the South American *T. rangeli* and an uncharacterised species *T.* sp H25 from Australian kangaroos and possums (Noyes *et al*. 1999, Hamilton *et al*. 2007, Paparini *et al*. 2011). The finding that the Australian *T.* sp H25 was at the periphery of the *T. cruzi* clade more than one decade ago raised questions about the diversification and dispersion of trypanosomes worldwide and led to the southern super-continent hypothesis to explain the evolution of *T. cruzi* clade trypanosomes (Figure 1) (Stevens, and Gibson. 1999a, Stevens *et al*. 1999b).

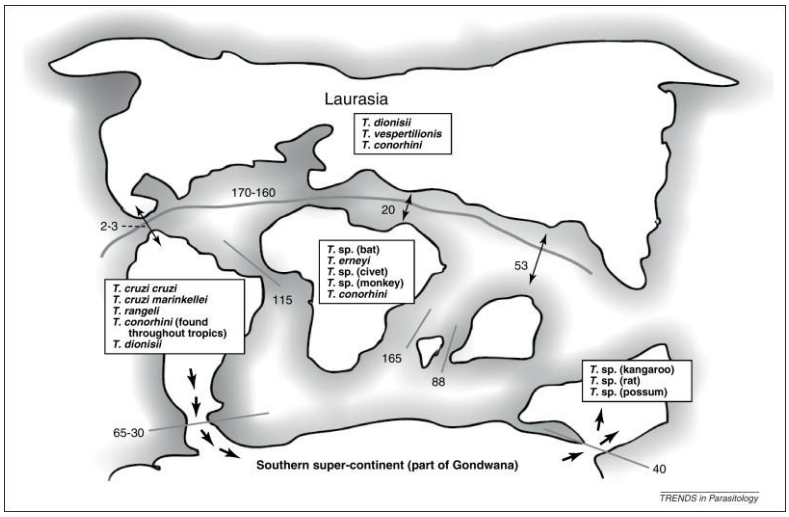


Figure 1. Southern super-continent or Gondwana hypothesis and distribution of *T. cruzi* clade genotypes. Grey lines indicate dates of splits between landmasses. Double-headed arrows indicate dates of collision between continents. Single-headed arrows indicate the direction of spread of marsupials to Australia from South America via Antarctica. All numbers are millions of years ago (Hamilton *et al*. 2012).

The “Gondwana” or the “Southern-super continent” hypothesis which has dominated the discussion of *T. cruzi* evolution in the past decade, suggested that *T. cruzi* and related parasites emerged from trypanosomes present in marsupials a long time ago when South America, Antarctica and Australia were joined in a southern-super continent, known as the Gondwana. After the separation of Gondwana, trypanosomes evolved in isolation in the mammals of South America, Antarctica and Australia, and old world bat trypanosomes within this clade were suggested to be the product of bat movements to South America, probably by virtue of the mobility of their hosts (Stevens, and Gibson. 1999a, Stevens *et al*. 1999b). More recently, the findings of many other trypanosomes from Australian wildlife that clade with trypanosomes from all over the world including *T.* sp ABF (wallaby) in the *T. theileri* clade, *T.* sp AAT (currawong) in the avian clade, and *T. binneyi* (platypus) and *T. chelodina* (tortoise) in the aquatic clade questioned the accuracy of this hypothesis (Hamilton *et al*. 2005a and b). The finding of several Australian trypanosomes that are closely related to trypanosomes outside Australia indicates that although the mammals of Australia have been geographically isolated, their trypanosomes have not (Hamilton *et al*. 2012). Furthermore, the discovery of two trypanosomes from a palm civet and a monkey from Africa in the *T. cruzi* clade (Hamilton *et al*. 2009) provided new evidence that did not support the southern-super continent hypothesis, and a new hypothesis was raised known as the “bat seeding hypothesis”. This hypothesis suggests that trypanosomes from the *T. cruzi* clade were originally bat parasites from the new world that evolved from within a larger clade of bat trypanosomes and subsequently made the switch into terrestrial mammal hosts in both the new and old worlds (Figure 2) (Hamilton *et al*. 2012).

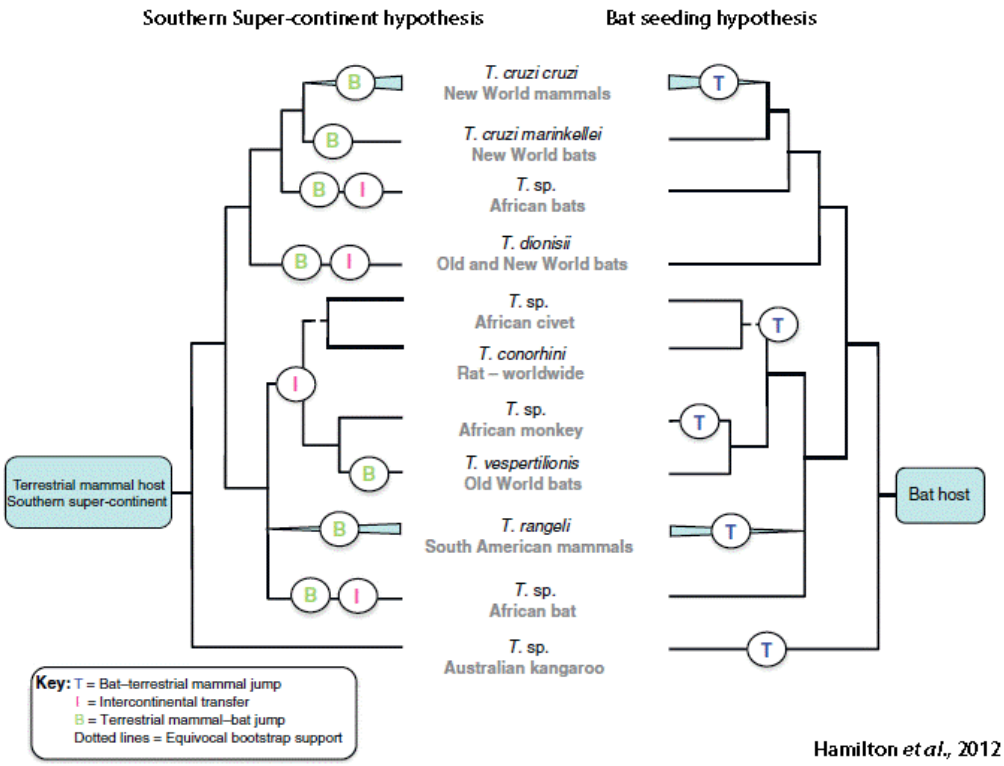


Figure 2. Hypotheses about the evolution of *T. cruzi* clade trypanosomes (Hamilton *et al.* 2012).

Hopefully, advances in genomic sequencing will facilitate the discovery of new *Trypanosoma* species and the description of uncharacterised species around the world. The discovery of new *Trypanosoma* species might provide important clues to a better understanding of the evolution of trypanosomes.

1.5 Morphology and life cycle

Trypanosomes are transmitted to the vertebrate host by haematophagous arthropods of the order Hemiptera (Triatomine bugs), Diptera (flies) and Siphonaptera (fleas) (Hoare. 1972). African trypanosomes for example, are transmitted by tsetse flies and American

trypanosomes by reduviid bugs. Insects from the class Arachnida (ticks and mites) have been incriminated as well in the transmission of different species of trypanosomes such as *T. thylacis,* and *T. copemani* (Mackerras. 1959, Austen *et al*. 2011). More recently, different leech species have been suggested to play a role in the transmission of *T. rotatorium*, *T. boissoni*, *T. triglae,* all trypanosomes of aquatic vertebrates (Lukeš *et al*. 1997), and have been suggested as well as the possible vector of *T. binneyi,* a trypanosome from platypuses, *T. chelodina* from Australian aquatic tortoises, and *T. cyclops*-clade trypanosomes from the frog *Mixophyes fleayi* and wallabies from Australia (Hamilton *et al*. 2005a).

Transmission occurs when arthropods, while feeding from host blood, take up the trypomastigote, which is the bloodstream form of the parasite. After this, trypanosomes undergo several transformations in form in the intestinal tract of the insect, ranging from epimastigotes in the gut to the production of metacyclic trypomastigotes, which are the infective forms of the parasite and are transmitted to a new vertebrate host by two different methods according to their localisation in the body of the insect vector (Hoare. 1972).

The Stercolarian group refers to trypanosomes such as *T. cruzi*, *T. lewisi*, and *T. theileri*, in which metacyclic trypomastigotes are located in the epithelium of the rectum of the insect. Transmission occurs when the infective forms are released in the urine or faeces near a bite site after feeding on a mammalian host. The parasite penetrates the vertebrate skin through the bite site or through adjacent mucous membranes by contamination. The Salivarian group refers to trypanosomes where metacyclic trypomastigotes are localised in the salivary glands of the insect and includes all trypanosomes from the

T. brucei complex. Transmission occurs when the insect injects the infective forms of the parasite while feeding from the bloodstream of the vertebrate host (Hoare. 1972). Trypanosomes can also be transmitted mechanically (ingestion-oral transmission or in blood transfusions) and vertically (from mother to progeny) (Leiby *et al*. 1999, Roellig *et al*. 2009, Kribs-Zaleta. 2010).

In the vertebrate host, the trypanosome life cycle involves two main strategies of parasite replication, either intracellularly in tissue cells or extracellularly in blood. In *T. cruzi*, for example, trypanosomes divide intracellularly in tissue cells. The life cycle begins when metacyclic trypomastigotes released in faeces of the vector penetrate the skin or mucosal membranes of the vertebrate host. Trypomastigotes then enter the host bloodstream (blood trypomastigotes) and migrate to different host tissues where they invade cells. Inside cells, trypomastigotes transform into amastigotes (intracellular stage) that divide continuously and differentiate again into trypomastigotes. This continuous replication of parasites and transformation of amastigotes into trypomastigotes leads to the lysis of invaded cells and release of trypomastigotes that initiate several new cycles of cellular invasion. The life cycle is completed when a new insect feeds on blood of an infected host (Figure 3). *T. cruzi* has been found infecting several tissues in humans and marsupials (De Souza *et al*. 2010) and *T. dionissi* and *T. erneyi*, both trypanosomes of bats, are able to infect host tissues as well (Baker *et al*. 1972, Lima *et al*. 2012).

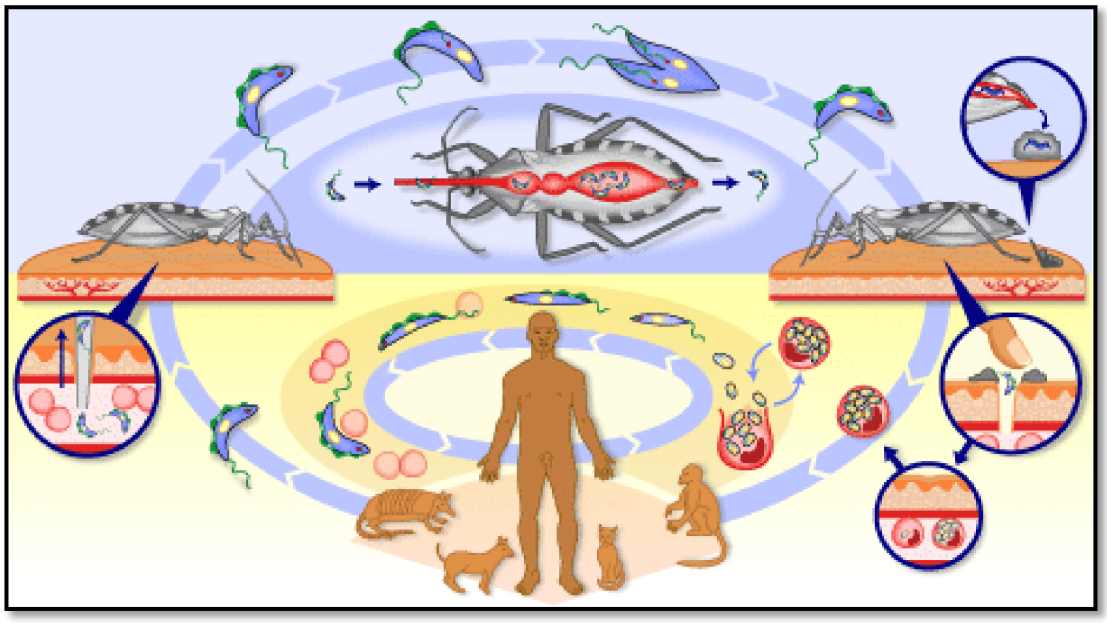


Figure 3. *T. cruzi* life cycle (http://www.who.int/tdr/diseases-topics/chagas/en/)

In contrast, all trypanosomes from the *T. brucei* complex lack the intracellular replicative form in their life cycle (the amastigote). Instead, they replicate in blood as trypomastigotes. The life cycle begins when infective metacyclic trypomastigotes in the salivary glands of tsetse flies are transmitted while feeding from the bloodstream of the vertebrate host. Trypomastigotes then enter the host bloodstream (blood trypomastigotes) where they replicate by binary fission. It has been demonstrated that trypanosomes from this group can be found extracellularly in the capillaries of some host tissues (Tabel *et al*. 2008). The life cycle is completed when a new insect feeds on blood of an infected host (Figure 4).

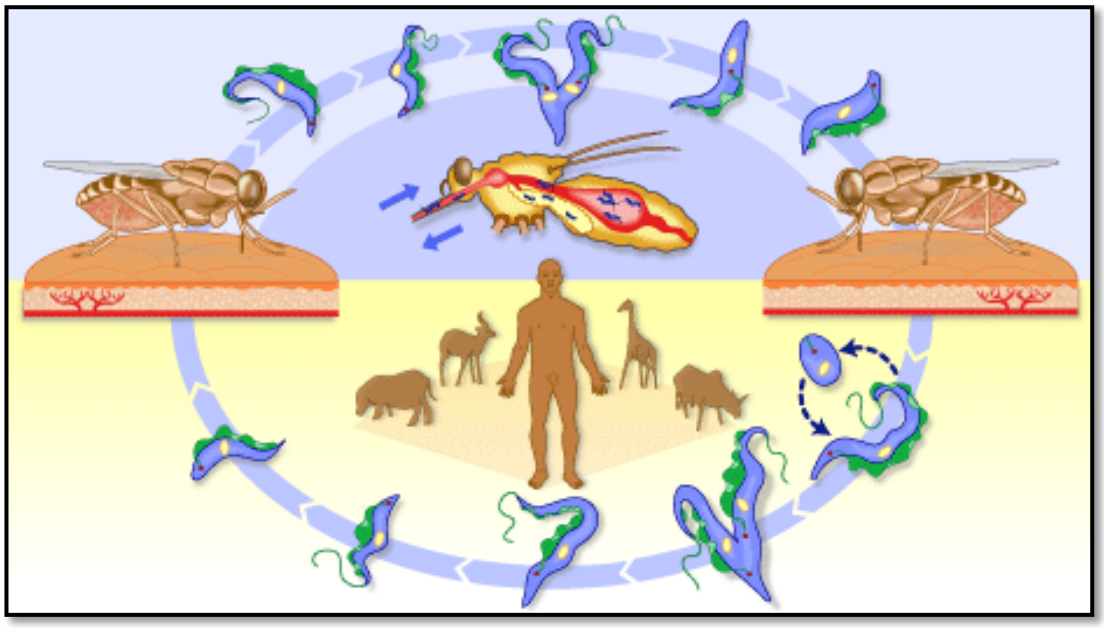


Figure 4. *T. brucei* life cycle
(http://www.who.int/tdr/diseases-topics/african-trypanosomiasis/en/)

The main developmental changes in morphology that trypanosomes undergo in their life cycle, in both the vertebrate and invertebrate host, have been defined by the position of the kinetoplast and the length of the flagellum (Field *et al*. 2009, Wheeler *et al*. 2013). The bloodstream trypomastigote presents a flagellum that emerges through a flagellar pocket near the rear end of the body and that is attached to an undulating membrane that runs the whole length of the cell. In this form, the kinetoplast is found in a posterior position relative to the nucleus, which is located in the middle of the cell (Figure 5). The amastigote, the intracellular stage in host tissues, presents a round/oval shape with no protruding flagellum and with a kinetoplast located in an anterior position relative to the nucleus (Figure 5). The epimastigote, the replicative stage in the intestinal tract of the insect vector, presents a kinetoplast located anterior and adjacent to the nucleus and a flagellum that emerges in the middle of the cell (Figure 5) (Field, and Carrington. 2009).

This stage of the parasite grows easily in culture media such as Grace's, LIT (Liver infusion tryptose), and Schneider's media, which contain several nutrients that simulate the environment in the intestinal tract of the insect. The spheromastigote presents a more rounded morphology and can be found either in the invertebrate or vertebrate host. The kinetoplast in the spheromastigote is located anterior and adjacent to the nucleus and its flagellum emerges in the middle of the cell as well as in the epimastigote (Figure 5).

Although in Australia many species and genotypes of trypanosomes and their hosts have been described, a complete lack of information about host-parasite relationships and life cycle is evident. To date, only one study has investigated the possibility that Australian trypanosomes could infect and replicate inside host cells *in vitro* and *in vivo* (Noyes *et al*. 1999). *In vitro* experiments showed that *T.* sp H25 isolated from a kangaroo and *T.* sp H26 isolated from a wombat were able to grow with *LLCMK1* cells in culture, but they did not invade or attach to the cells. Moreover, *in vivo* experiments showed the absence of parasites in blood, spleen and liver of mice experimentally infected with both trypanosomes four weeks post-infection (Noyes *et al*. 1999).

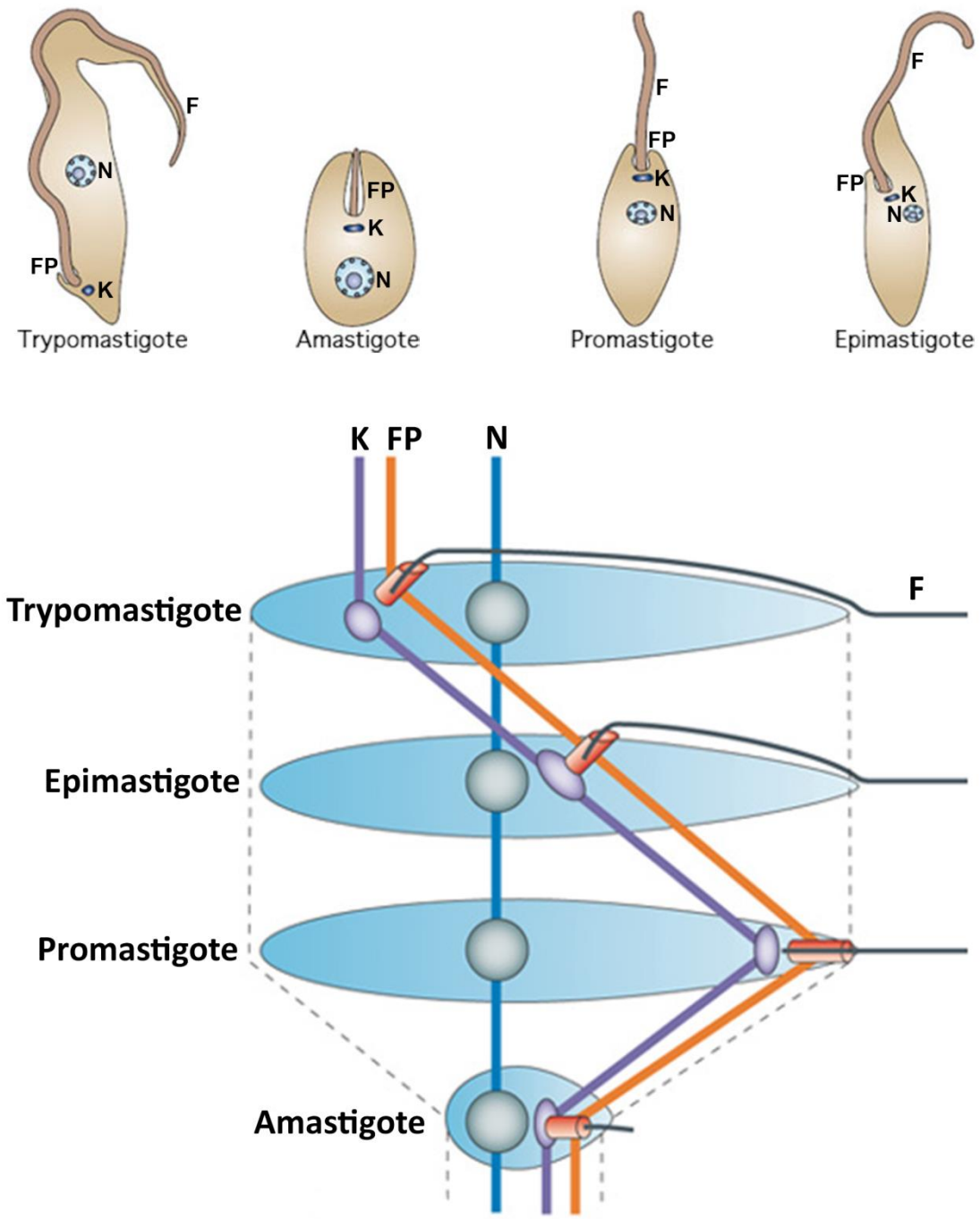


Figure 5. Morphology of all developmental stages of trypanosomes in both the vertebrate and invertebrate host. N: nucleus, K: kinetoplast, F: flagellum, FP: flagellar pocket. Image modified from Field, and Carrington (2009).

1.5.1 The unique organelle "the kinetoplast"

Parasites from the genus *Trypanosoma* are flagellate protozoans that belong to the order Kinetoplastida (Honigberg. 1963), which are characterised by the presence of a unique organelle termed the kinetoplast. This organelle is a modified mitochondrion localised at the basal part of the flagella, which encloses the mitochondrial DNA of the cell (Simpson. 1972). The kinetoplast DNA or kDNA represents more than 10% of the total cellular DNA and presents a peculiar genomic organization unlike that of any other DNA in nature (Riou *et al*. 1969). It consists of two types of circular DNA molecules, maxicircles and minicircles, which can be topologically interlocked into a single and massive network according to the species (Shapiro *et al*. 1995b). The size of maxicircles usually ranges from 20 to 40 kb and they are present in a few dozen identical copies per cell. In contrast, minicircles are present in several thousand copies per cell and are usually nearly identical in size ranging from 0.5 to 10 kb depending on the species (Lukeš *et al*. 2002). Maxicircles encode typical mitochondrial gene products, such as cytochrome c oxidase subunits I, II and III (COI, COII and COIII) and apocytochrome b (CYb), but, remarkably, some of the protein-coding genes are encrypted. To generate functional mRNAs, the cryptic maxicircle transcripts undergo postranscriptional modification via an intricate RNA editing process that requires insertion and deletion of uridine residues at specific sites in the transcripts. Minicircles and some maxicircles encode guide RNAs (gRNAs) that provide the genetic information for editing (Maslov *et al*. 1994).

Kinetoplastids are divided into two groups, trypanosomatids that are obligate endoparasites such as trypanosomes, and bodonids that are free-living protozoans (Moreira *et al*. 2004). Interestingly, a huge diversity in kDNA structure and

conformation has been demonstrated in both free-living and parasitic species, and several studies have addressed the biological significance of this, and the role of kDNA in parasitism. A disk-shaped and highly condensed kDNA network has been reported in *T. cruzi*, *T. brucei, L. tarentolae* and *C. fasciculate* (Figures 6A and B) (Shapiro *et al*. 1995). This type of kDNA structure consists of maxicircles and minicircles organised in a condensed network with a disk-shaped structure. Minicircles are stretched out and aligned side-by-side almost perpendicular to the planar face of the disk, and constitute about 90% of the mass of the kDNA, while maxicircles constitute only about 10% (Valencia. 2014). The thickness of the disk corresponds to about half of the circumference of each minicircle and both the disk thickness and the size of minicircles have been shown to differ between species (Lukeš *et al*. 2000, Shapiro, and Englund. 1995). The main characteristic of the kDNA network organization is that minicircles within the network are catenated. This was previously demonstrated *in vitro* using the enzyme topoisomerase II, which in nature modulates DNA topology. Covalently closed catenated minicircles are released from the central zone of the network through decatenation by the action of type II DNA topoisomerase enzymes (Drew *et al*. 2001) prior to their replication (Shapiro, and Englund. 1995). In contrast, the minicircles in species of the family Bodonidae such as *Bodo caudatus* and others are not organised in a network (non-catenated) (Hajduk *et al*. 1986). Instead, they are distributed in diverse forms across the mitochondrial matrix known as Poly-kDNA, Pan-kDNA, Mega-kDNA, and pro-kDNA (Vickerman. 1990, Lukeš *et al*. 2002). Pro-kDNA is found in the free-living *Bodo saltans*. Its kDNA is organised in a bundle-like structure that is usually composed of individual non-catenated 1.4 kb minicircles (Figure 6C) (Blom *et al*. 2000). However, *Dimastigella trypaniformis* (a commensal of the intestine of a termite), *Dimastigella mimosa* (a free-living bodonid isolated from a sewage plant), and *Cruzella*

marina (a parasite of the intestine of a sea squirt) present a kDNA organization distinct from that seen in *B. saltans*. Instead of being condensed into a single globular bundle, their kDNA is distributed among various discrete foci throughout the mitochondrial lumen known as poly-kDNA (Figure 6E and F) (Breunig *et al*. 1993). The kinetoplast DNA of *Cryptobia helicis*, a parasite of the receptaculum seminis of snails, fills most of the mitochondrial matrix and is known as Pan-kDNA (Figure 6D) (Lukeš *et al*. 1998). Mega-kDNA is the most unusual kDNA organization and is present in the fish parasite *Trypanoplasma borreli*. The kDNA of this fish parasite is distributed fairly uniformly throughout a large region of the mitochondrial matrix (Figure 6E and F). Molecular studies have demonstrated that Mega-kDNA does not contain minicircles at all (Maslov, and Simpson. 1994).

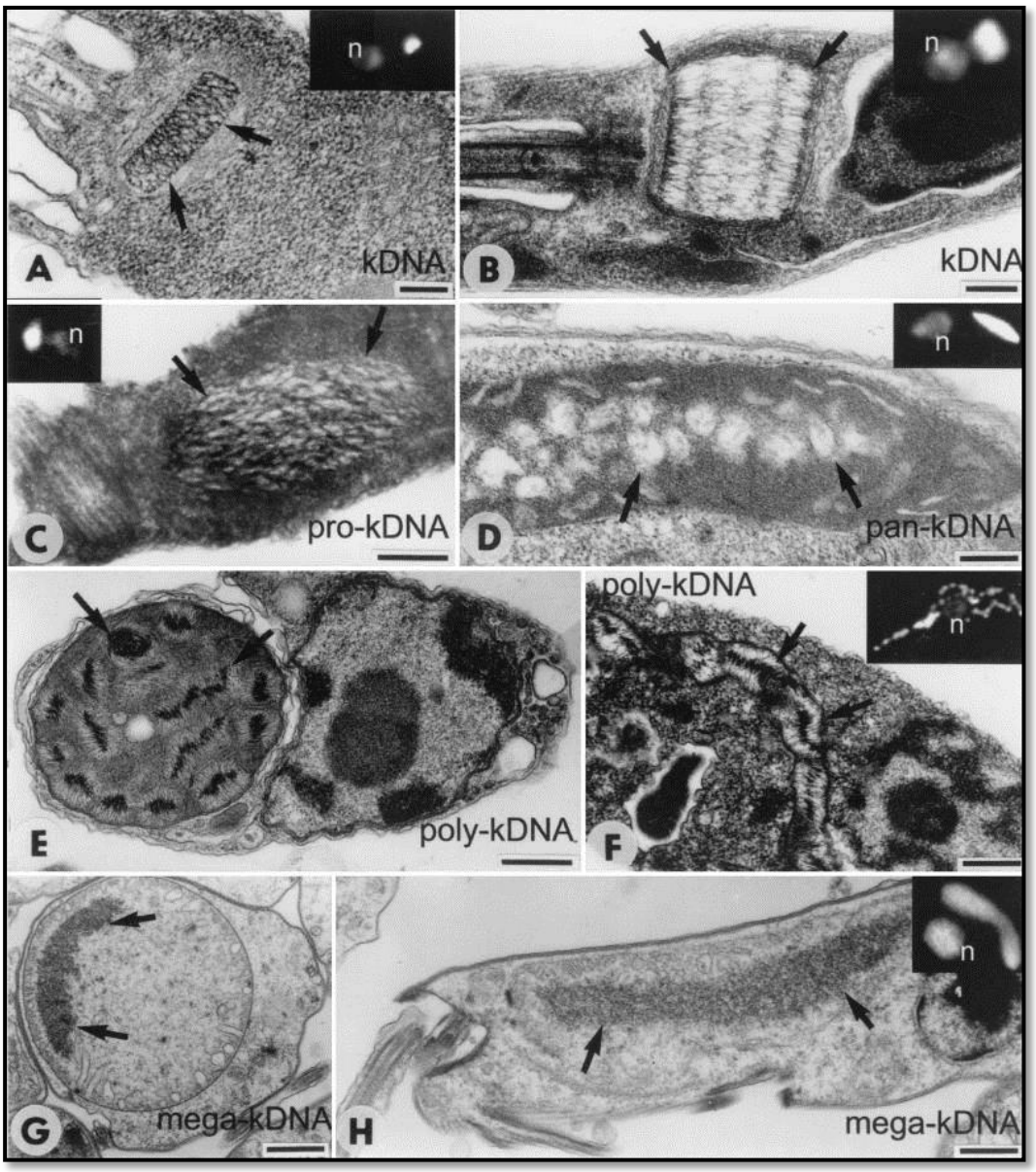


Figure 6. Electron microscopy images of the kinetoplast of different kinetoplastids. Arrows in electron micrographs indicate kDNA. Insets show DAPI-stained cells (n: nucleus) - kDNA is stained brightly. A: classical disk-shaped kDNA of *C. fasciculata*; B: kDNA disk of *T. avium*; C: Pro-kDNA bundle of *B. saltans*; D: Pan-kDNA of *C. helicis*; E and F: mitochondrion of *D. trypaniformis*; G and H: *T. borreli* mega-kDNA. Scale bars: 200 nm in panels A to F, and 1 μ m in panels G and H (Lukeš *et al*. 2002).

The kinetoplast has been of major interest not only because of its peculiar mitochondrial genome organisation, but also because its heterogeneity between species can be a clue to understanding the evolutionary history of trypanosomatids (Simpson *et al*. 2002). Phylogenetic studies have shown that kinetoplastids with a non-network organization of their kDNA, represent early branches of the kinetoplastid tree (Fernandes *et al*. 1993, Lukeš *et al*. 1997, Maslov, and Simpson. 1994, Blom *et al*. 1998). In contrast, the kDNA of late branching trypanosomatids including *T. cruzi*, *T. brucei* and *Leishmania* is organised in a network (Figure 7). It has been suggested that since more recently evolved trypanosomes have networks, there is presumably some advantage to the network structure (Chen *et al*. 1995). The pattern of arrangement of kinetoplastids in phylogenetic trees makes them interesting objects for the study of the evolutionary history of parasitism.

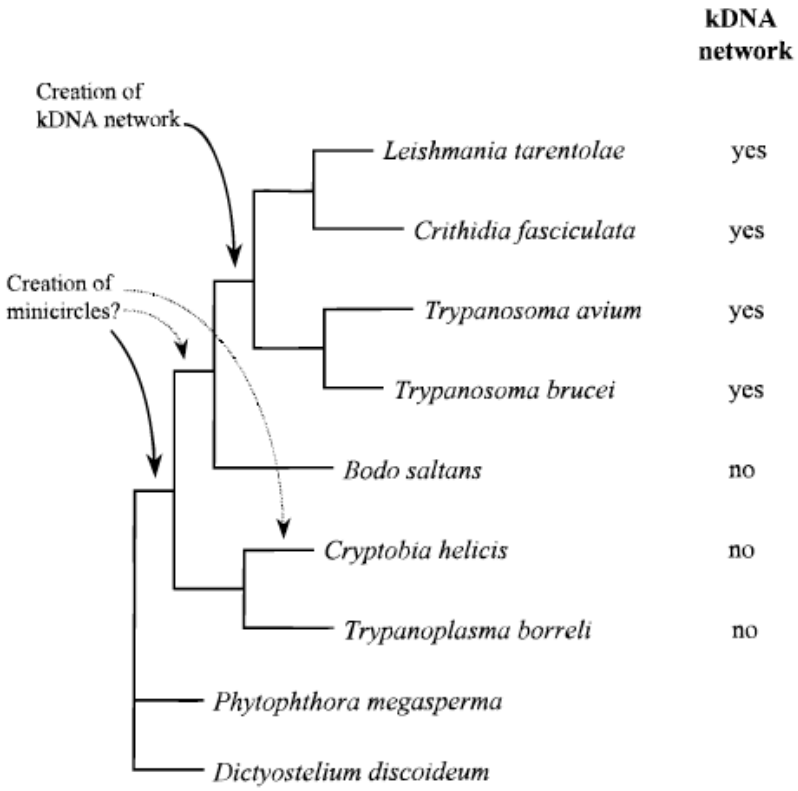


Figure 7. Kinetoplastids phylogenetic tree based on the small-subunit rRNA (Lukeš *et al*. 2002)

In addition to differences in the ultrastructure of the kDNA between different kinetoplastids, variation in size, number and sequence organization of the minicircles has also been reported. One of the most studied networks is that of *Crithidia fasciculata*. Its kDNA network contains approximately 5,000 minicircles of 2.5 kb each, in which each and every minicircle is interlocked with three of its neighbours (Lukeš *et al*. 2010). In contrast, *T. cruzi* contains approximately 10,000 to 20,000 minicircles of 1.4 kb each (Avila *et al*. 1995). *T. lewisi, T. brucei* and *Leishmania tarentolae* minicircles are approximately 1.0 kb each (Simpson. 1987). Sequencing revealed that the minicircles of most kinetoplastids are heterogeneous in sequence. They have one or more conserved regions (roughly 100 to 200 base pairs) that contain regulatory and initiation sequences of replication (Hines *et al*. 2008). A common feature within the conserved region of the minicircles is the presence of a 12-nucleotide sequence named "The Universal Minicircle Sequence" (UMS) that is conserved in most trypanosomatids and is the initiation site for leading-strand synthesis (Ray. 1989). However, the number of the UMS elements and their location in each minicircle differ within species (Ponzi *et al*. 1984, Sugisaki *et al*. 1987, Degrave *et al*. 1988). The minicircles of both *C. fasciculata* and *T. lewisi* contain two UMS elements located 180 degrees apart (Ponzi *et al*. 1984, Sugisaki, and Ray. 1987), whereas those from *T. brucei* and *Leishmania tarentolae* contain only one (Kidane *et al*. 1984, Jasmer *et al*. 1986). In contrast, *T. cruzi* minicircles contain four conserved regions present as direct repeats located 90 degrees apart (Degrave *et al*. 1988). Figure 8 shows the size of the minicircles and number of conserved regions (UMS) of different species of trypanosomes. The regions of the minicircles flanked by the different UMS elements are heterogeneous in sequence and have been used to investigate intraspecific variations within species (Telleria *et al*. 2006). Due to the minicircle abundance and heterogeneity in sequence, they have been

widely used in the development of sensitive and specific diagnostic molecular tools using PCR (Noyes *et al*. 1998, Botero *et al*. 2010, Ceccarelli *et al*. 2014).

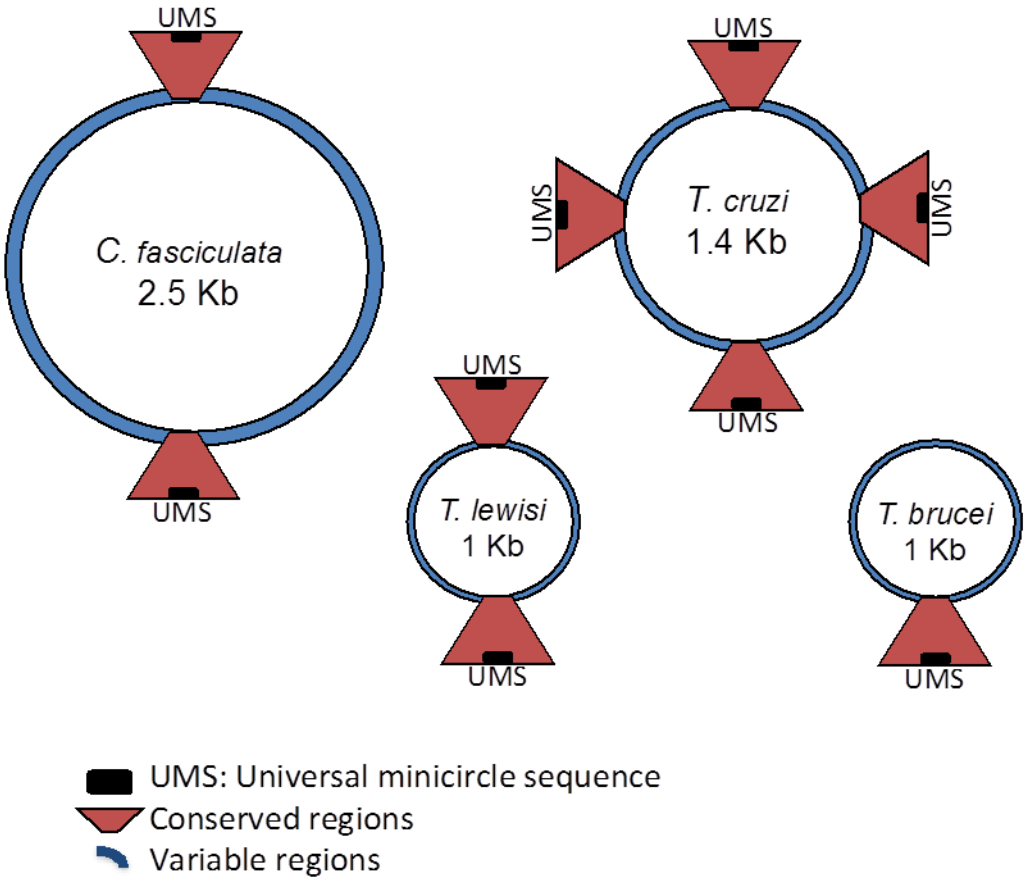


Figure 8. Differences in the minicircle sizes and in the number and position of the conserved regions (UMS) from different species of trypanosomatids.

The UMS is also the specific binding site for the UMS-Binding Protein (UMSBP), a protein involved in kDNA replication (Tzfati *et al*. 1995). The UMSBP is located in two sites in the kineto-flagellar zone (KFZ), where minicircle replication initiates (Abu-Elneel *et al*. 2001). The UMSBP of *C. fasciculata* for example, has been extensively studied (Onn *et al*. 2006). Recent studies using antibodies raised against *C. fasciculata*

UMSBP have found the presence of *C. fasciculata* UMSBP homologues in other trypanosomatids such as *T. cruzi* and *T. brucei* (Coelho *et al*. 2003, Milman *et al*. 2007). It has been shown that the knockdown of the UMSBPs by RNAi (RNA interference) not only affects the initiation of minicircle replication, but also inhibits segregation of the daughter networks and blocks nuclear division (Milman *et al*. 2007), suggesting this protein as a potential powerful drug target, and thus revealing a broader role for the UMSBP than originally thought.

1.6 Pathogenicity and trypanosomes associated with wildlife declines and extinction

Trypanosomes range from non-pathogenic species to those that are highly pathogenic and are the causative agents of many diseases of medical and veterinary importance (Hoare. 1972). One of the most important pathogenic trypanosomes is *T. cruzi*, the agent of Chagas disease in humans, that also affects domestic and wildlife animals. Its pathogenicity is attributed to the damage of several vital organs in the vertebrate host caused by a strong immune response triggered by the presence of the parasite in tissues (Zhang *et al*. 1999). Infections consist of an acute initial phase characterised by high parasitaemias in blood in the absence of clinical manifestations; however, acute Chagas disease can led to a rapid death of the host. Subsequently, the disease progresses to a chronic phase that typically begins with a long period of latency (indeterminate chronic form) characterised by the presence of *T. cruzi* amastigotes in several tissue cells where they can persist for years in the absence of inflammatory reactions and therefore without any significant damage to the host (Teixeira *et al*. 2006, Teixeira *et al*. 2011a). However, about 30 percent of the indeterminate chronic infections progress over years to clinically evident chronic disease. This clinically evident phase of the disease includes diverse manifestations that involve vital organs such as heart, spleen, liver, intestine,

and nervous system. The persistence of parasites in the cytoplasm of these tissue cells, and their release after the lysis of cells triggers a strong inflammatory immune response that results in tissue damage and necrosis. The most common chronic form of the disease is when the parasite affects the heart in a process called chagasic cardiomyopathy. Some areas of the heart can be severely damaged and are characterised by inflammatory infiltrates, necrosis and the progressive deposition of fibrotic tissue. However, inflammation and lesions might increase over time reaching the entire heart simultaneously and leading to heart failure and death of the host (Teixeira *et al*. 2006). In contrast, the mortality rate in the acute phase is below 10%, and is attributed to high parasite burdens that cause cardiac failure, meningitis, or encephalitis (Teixeira *et al*. 2011b). Although limited research has been conducted regarding the pathogenicity of *T. cruzi* in wildlife, a few studies have shown that this parasite is also able to cause pathology in marsupials. Naturally infected *D. marsupialis* exhibited hearts with myocarditis characterised by mononuclear cell infiltrates and target cell lysis; skeletal and smooth muscles of the oesophagus and the small and large intestines presented strong inflammatory infiltrates as well (Teixeira *et al*. 2001). Furthermore, the presence of intracellular amastigotes and inflammatory infiltrates of moderate to severe intensity in scent glands and hearts of the same marsupial species has also been demonstrated (Carreira *et al*. 1996).

In contrast, the pathogenicity of trypanosomes from the *T. brucei* complex leads to their ability to undergo rapid surface membrane variation in a process that is known as antigenic variation. During this process, African trypanosomes change their membrane proteins in order to evade the host immune system, and multiply with every surface modification at a more rapid speed than the immune system can build up a defence

against it (appropriate antibodies) (Berriman *et al*. 2005). A healthy immune system can regularly produce antibodies in a reasonably brief period, but not fast enough to destroy the trypanosomes before their protein compositions are modified. Human or animal African trypanosomiasis has two clinical stages. The first corresponds to the multiplication of trypanosomes in the blood and lymphatic system, which often goes undiagnosed. When the parasites cross the blood-brain barrier, the disease progresses to the second stage, which is characterised by neurological symptoms and, without treatment, evolves towards body wasting, somnolence, coma, and death. The pathologic manifestations caused by *T. brucei* and other pathogenic African trypanosomes include anaemia, immune complex disease, progressive destruction of lymphoid organs and other tissues, reduced fertility, cachexia and neurologic disorders (WHO 2010). In Africa, the major pathogenic species of trypanosomes from domestic ruminants and wildlife are *T. congolense, T. vivax, T. b. brucei* and *T. evansi*.

Experimental infections have been carried out and have demonstrated that a number of wildlife species are susceptible to *T. evansi*. Donkeys infected with a Brazilian strain of *T. evansi*, originally isolated from a naturally infected dog (Aquino *et al*. 1999), developed a chronic disease. The course of infection was characterised by a marked decline in haemoglobin, packed-cell volume, and erythrocyte count, leading to anaemia after successive peaks of parasitemia. All infected donkeys exhibited enlargement of spleen and lymph nodes and congestion of the lungs (Cadioli *et al*. 2006). *T. evansi* experimentally infected *Bandicota bengalensis* developed an acute disease course leading to premature death of the animal - the histological changes seen in liver, spleen, lung, kidney, and heart comprised inflammatory, degenerative, and necrotic changes, similar to those seen in *T. evansi* infections in natural hosts, and in human sleeping

sickness (Biswas *et al*. 2001). Furthermore, in attempts to test the potential for the spread of *T. evansi* in Papua New Ghinea and Australia, Agile wallabies (*Macropus agilis*) and dusky pademelons (*Thylogale brunii*) were experimentally infected with this parasite. Both species were susceptible to the infection developing high parasitaemias that persisted until death. Animals exhibited clinical signs such as anorexia, weakness, ataxia, and anaemia, and autopsies revealed pericarditis, splenomegaly and ulcerative gastritis and enteritis (Reid *et al*. 2001).

Some trypanosomes, normally considered as non-pathogenic, are capable of inducing detrimental effects in the host when encountering new or naïve host species following their introduction into a new habitat (Maraghi *et al*. 1989). This was the case with *T. lewisi,* a non-pathogenic trypanosome of rats that was involved in the extinction of an endemic murid species on Christmas Island at the beginning of the 20th century (Wyatt *et al*. 2008). This parasite has a limited antigenic variation, and infections are usually self-limiting leading to the eradication of circulating parasites and generating protection against reinfections (D'Alessandro *et al*. 1991). However, the infection with this parasite in native rats on Christmas Island appeared to behave differently. Black rats (*Rattus rattus*), a common reservoir of *T. lewisi*, were inadvertently introduced onto the island in 1899 as a result of increased ship traffic. Within 5 years after their introduction, the population of two endemic murids, the Christmas Island rat (*Rattus macleari*) and the bulldog rat (*Rattus nativitatis*), declined abruptly and were considered extinct by 1908. Interestingly, individuals were described with an abnormal behaviour (nocturnally active rodents appearing during the daytime), and were infected with trypanosomes (Durham. 1908). A more recent study that examined museum specimens collected from the introduced *R. rattus* and from both native species *R. macleari* and

R. nativiatis during the extinction period, confirmed they were infected with *T. lewisi*. Furthermore, analysis of blood samples taken from the bulldog rat before the black rat was introduced showed absence of infection with trypanosomes (Wyatt *et al*. 2008). The results obtained showed a correlation between the extinction of the native rat on Christmas Island and the arrival of *T. lewisi* in black rats suggesting that this parasite could be the cause of the extinction. It was also suggested that both endemic rats were immunologically naïve and therefore were highly susceptible to *T. lewisi* infections. It is also important to note that other mammals declined on the island after the extinction of *R. macleari*. The only other native ground-dwelling mammal on the island, the Christmas Island shrew (*Crocidura trichura*), survived many decades after the disappearance of the endemic rodents, but it has not been seen since 1985 despite considerable surveying efforts (Schulz *et al.*, 2004). More recently, the Christmas Island pipistrelle bat (*Pipistrellus murrayi*) appears to have become extinct in 2010 (Martin *et al*. 2012). The relationship between the extinction and decline of these species just after the loss of the endemic *R. macleari* and *R. nativiatis,* and the presence of *Trypanosoma* needs to be investigated, especially considering the reported low white blood cell counts in this bat species (Lumsden *et al*. 2007) that could be associated with infections with trypanosomes.

Host stress and concurrent infections might exacerbate the detrimental effects caused by *Trypanosoma* infections, even during infections with trypanosomes that are normally considered as non-pathogenic (Brown *et al*. 2000). Stress generated by captivity was suggested as a significant underlying factor in the sudden death of various platypuses infected with trypanosomes. Pathological findings in tissue sections from lung, liver, and heart from platypuses held in captivity from one to three weeks, showed marked

inflammation and tissue necrosis associated with trypanosome infection (McColl. 1983). Moreover, it was shown that the depression of the host immune system caused by stress and/or concurrent infections with *T. theileri* could increase parasitemias and facilitate the dispersion of *T. theileri* through several organs and the central nervous system in cattle (Ward *et al*. 1984, Hussain *et al*. 1985, Seifi. 1995, Braun *et al*. 2002, Villa *et al*. 2008).

A few species of trypanosomes are known pathogens of various amphibians (Wright *et al*. 2006). The population of the North American Eastern hellbender salamanders (*Cryptobranchus alleganiensis*) is declining and was recently classified as an endangered species by the US Fish and Wildlife Service (Register. 2011). Consequently, many investigations have evaluated their health status including the examination of blood cell counts, heavy metal loads, stress hormone levels, immunological responses, disease testing, and blood parasite prevalence (Solís *et al*. 2007, Huang *et al*. 2010, Hopkins *et al*. 2011). Interestingly, a high prevalence of infection of 56.3% with *T. cryptobranchi,* was found during a recent health survey, suggesting the need for further studies to determine if this *Trypanosoma* species is spreading among hellbender salamanders and whether their presence solely or in conjunction with other microorganisms are adversely influencing their health and survival (Davis *et al*. 2013).

Experimental infections have been used frequently to overcome the difficulties in the evaluation of pathogenicity of trypanosomes in naturally infected wildlife. Histopathological lesions in heart and spleen, characterised by focal myocarditis and pericarditis and the presence of chronic mononuclear inflammatory cells were reported in spleen of canaries experimentally infected with the avian *T. bouffardi* (Molyneux *et*

al. 1983). Carp fingerlings experimentally infected with *T. danilewskyi* exhibited infiltrative and proliferative changes in the renal tissue, pancreas and in various connective tissues (Lom *et al*. 1986). Moreover, anaemia, altered blood parameters and anorexia were reported in goldfish and juvenile common carp with high levels of *T. danilewskyi* in blood (Islam *et al*. 1991, Ahmed *et al*. 2011).

Within Australia, little is known about the pathogenicity of native trypanosomes. To date, only one study has associated Australian trypanosomes with the poor health status of threatened wildlife. McInnes *et al.* (2011b) showed an association between *T. gilletti* infections with low blood packed cell volume values (PCV) and low body condition scores in koalas with signs of chlamydiosis, bone marrow disease or koala acquired immune deficiency syndrome (kAIDS) caused by infection with the koala retrovirus (KoRV). It was suggested that *T. gilletti* might have the ability to potentiate pathogenicity during concomitant infections in the koala (McInnes *et al*. 2011b). Reports of *Trypanosoma* infections in other threatened or endangered Australian native marsupials such as the woylie, Gilbert's potoroo, quokka, and wombat (Noyes *et al*. 1999, Smith *et al*. 2008, Austen *et al*. 2009) prompted the need for further studies that help to elucidate the potential pathogenicity of Australian trypanosomes and their impact on the health and decline of native wildlife species.

1.7 The critically endangered Australian marsupial "the woylie"

The woylie or brush-tailed bettong (*Bettongia penicillata*) is a small rat-kangaroo marsupial that was once distributed in most of the southern half of the Australian mainland, including the arid and semi-arid zones of Western Australia, the Northern Territory, South Australia, New South Wales and Victoria (Figure 9). However, by the

1960s, the geographical distribution of the woylie became confined to three locations in Western Australia (WA), the Upper Warren Region, Tutanning Nature Reserve and Dryandra Woodland (De Tores *et al*. 2008, Wayne. 2008). By the 1970s, woylies were listed as critically endangered due to a drastic reduction in abundance from habitat destruction and introduced predators (Orell. 2004, De Tores, and Start. 2008).

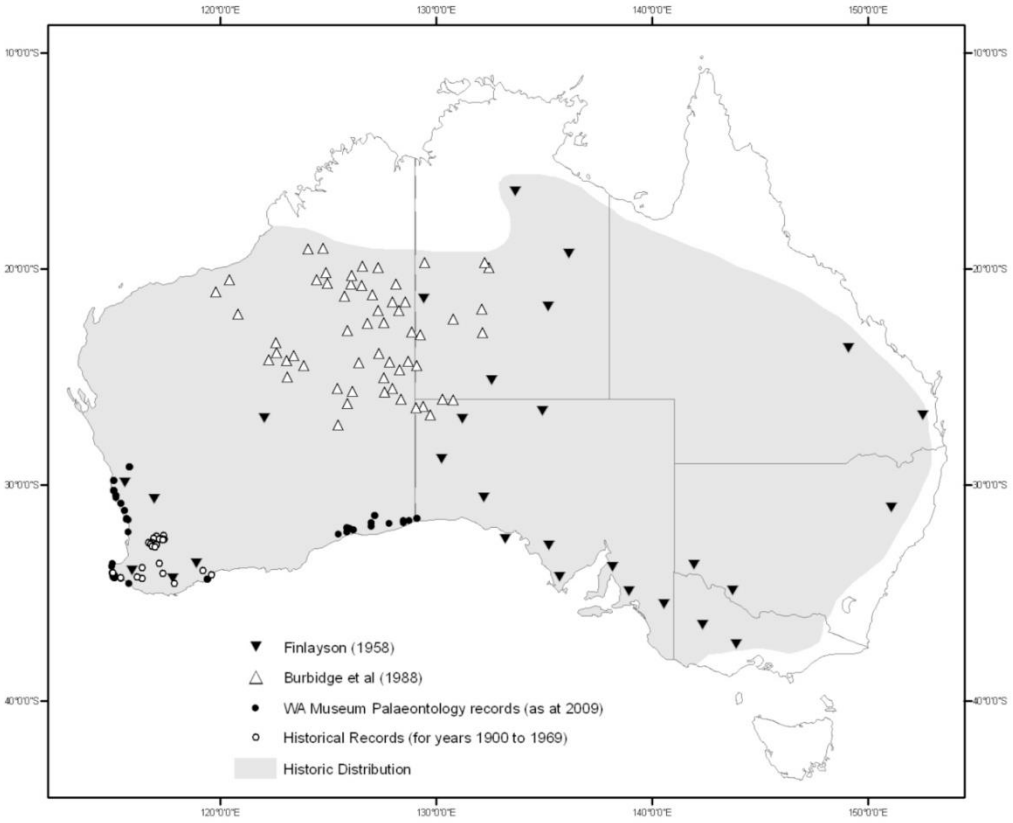


Figure 9. Historical distribution of the woylie in Australia (Yeatman *et al*. 2012).

Significant conservation efforts, which included predator control and captive breeding and release, led to a dramatic increase in abundance from the mid 1970s into the early 2000s. As a consequence of these efforts, the woylie was the first Australian mammal to be delisted from the Commonwealth and State conservation lists in 1996 (endangered/

threatened) (Start *et al*. 1998). However, since 1999, remaining populations have undergone a dramatic 90% reduction in abundance despite no apparent increase in the number or type of predators in the region and no obvious decrease in natural resources (Wayne *et al*. 2013a). The Tutanning population now appears to be extinct and the Upper Warren population, which constituted about 85% of the species in 1999, declined by 95% between 2002 and 2008 (Wayne *et al*. 2013a). The other natural population, Dryandra, has declined by 92% between 1999 and 2006. As a consequence of these population declines, woylies were included once again on the endangered species list (Wayne. 2008, Groom. 2010). Figure 10 shows the trend of the woylie decline in the Upper Warren region between 1998 and 2010.

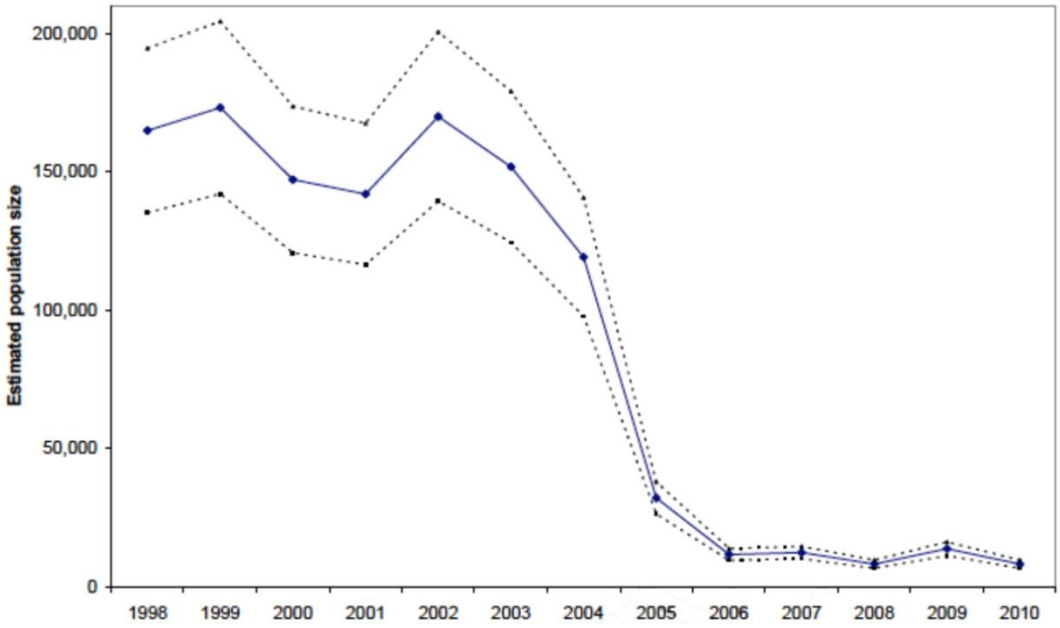


Figure 10. Woylie population sizes estimated for the Upper Warren region based on a conversion of median capture rate to density (R^2=0.90). The lower and upper 95% confidence intervals for the regression of co-efficient for the relationship between capture rate and density are presented as dashed lines. X-axis: years (Wayne *et al*. 2013a).

The cause of the second wave of the decline of the woylie is unknown, but, the fact that predation by foxes and cats were previously identified as key factors in the past woylie decline made them the first candidate to investigate (Wayne *et al*. 2013b). A broad-scale aerial baiting began in 1996 and is ongoing as part of the 'Western Shield' conservation program (Friend *et al*. 2004), and the monitoring of introduced predators also initiated across the Upper Warren region in 2006. Although, there was an average increase in fox activity since 2006, it was suggested that it might not be directly related to the woylie decline because the majority of the decline in the region occurred before this. Moreover, since 2011, fox activity has apparently not increased and has seemed to stabilise, and the regional average in cat activity, which initially increased since 2006, appears to have reduced slightly since February 2008 (unpublished data, woylie progress report 2010-2013). Human activity and habitat loss have also been suggested to be influencing the decline, although preliminary investigations have shown it is unlikely (Wayne. 2008).

Interestingly, the decline has spread around 4 kilometers per year through the Upper Warren region, leading to the hypothesis that this spatio-temporal pattern of the decline might be due to an agent with limited mobility, such as an infectious diseases moving through the woylie population (Wayne *et al*. 2013a). The fact that the rates of average decline (25–95% per annum) are greater than what would be expected in the event of a complete failure of recruitment by reproduction (average life expectancy of woylies is 4–6 years) (Christensen. 1995), suggested that the rapid and substantial woylie decline might be in part due to increased adult mortality (Wayne *et al*. 2013a). It has been previously shown that disease can affect conservation efforts, acting as a contributing threat in the endangerment of wildlife hosts, and causing severe population declines, such as the case with white-nose syndrome caused by *Geomyces* spp. fungus in bats,

parapoxvirus in red squirrels in the United Kingdom, and facial tumour in the Tasmanian devil (Blehert *et al*. 2009, Thomas *et al*. 2003, Jones *et al*. 2007). Woylies have been reported to be infected with different parasites including piroplasms from the genera *Babesia* and *Theileria* (Paparini *et al*. 2012, Rong *et al*. 2012), *Toxoplasma* (Parameswaran. 2008), and *Trypanosoma* (Smith *et al*. 2008, Averis *et al*. 2009, Paparini *et al*. 2011, Thompson *et al*. 2013, 2014a). A recent study investigating if infections with virus could be a contributing factor to the decline found that woylies have never been exposed to wallal and warrego orbiviruses, macropod herpesvirus 1, encephalo-myocarditis virus, alphaviruses Ross River and Barmah Forest, and flaviviruses Kunjin and Murray Valley encephalitis (Pacioni *et al*. 2014). The prevalence of infection with *Babesia* (6.2%) and *Toxoplasma* (3.61% from 271 woylies examined) were too low to be considered as major contributors to the decline (Parameswaran. 2008, Paparini *et al*. 2012). In contrast, the prevalence of infection with *Theileria* (80.4%) and trypanosomes was considerably high. However, microscopic evaluations of woylie blood smears infected with *Theileria* revealed no red cell injury or anaemia (benign infections) suggesting that this parasite might not be associated with the decline (Rong *et al*. 2012). No studies have investigated signs of virulence and pathogenicity caused by *Trypanosoma* infections in the woylie. Trypanosomes were initially detected in woylies in 1998, with an average prevalence of infections of 35% and it was suggested that infections with this parasite should be considered as an important factor influencing the decline (Smith *et al*. 2008). More recently, a higher prevalence of infection of 60% was reported in woylies from the same localities (Thompson *et al*. 2014a) suggesting the spread of trypanosomes through the woylie population. The fact that trypanosomes can be potentially pathogenic under certain circumstances, and that disease has been considered the most likely primary and

ultimate agent of decline (Wayne *et al*. 2013a) have prompted the investigation of trypanosomes as a substantial factor influencing the decline. Synergistic effects between parasites and the involvement of stressors that may trigger disease have also been considered. Despite all the efforts to preserve the species including the translocation of seven populations within its previous range, any substantial recovery of the woylie has not been sustained in wild populations (Wayne *et al*. 2013b). To date, the only substantial translocated population that has remained stable in Western Australia is a population at Karakamia wildlife sanctuary, a predator-proof fenced reserve located 50 km northeast of Perth. Confirming the causes of the decline and/or the factors limiting the woylie recovery remains a priority, and many efforts to preserve the species are still ongoing.

1.8 Trypanosoma drug therapies

Different drugs have been developed and are currently used to combat different *Trypanosoma* infections. However, they have been far from satisfactory due to the lack of complete efficacy, systemic toxicity, side effects, and intraspecific variation in susceptibility or resistance within *Trypanosoma* species.

In the case of Chagas disease, since the late 1960s to early 1970s, two nitroheterocyclic drugs have been available and are still currently used to treat *T. cruzi* infections: benznidazole and nifurtimox (Castro *et al*. 2006). Benznidazole (N-benzyl-2-nitro-1-imidazoleacetamide) is a nitroimidazole derivative, while nifurtimox (4[(5-nitrofurfurylidene) amino]-3-methylthiomorpholine-1,1-dioxide) is a nitrofuran derivative (Maya *et al*. 2007). Both drugs act through the formation of free radicals and/or electrophilic metabolites caused by the reduction of their nitro groups to an

amino group by the action of nitroreductases. These products inhibit the growth of the parasite (Maya *et al*. 2007). Benznidazole and nifurtimox have been shown to be curative only in acute or early chronic infections (Sgambatti de Andrade *et al*. 1996), and a decline in their efficacy has been reported in the late chronic phase of the infection (Sosa-Estani *et al*. 2006). The ineffectiveness of the drugs during the late chronic phase has been related to their incapacity to completely clear or inhibits the growth of *T. cruzi* amastigotes in tissues (Muelas-Serrano *et al*. 2002). Posaconazole, a triazole that targets the sterol 14alpha-demethylase enzyme (also known as CYP51), inhibit *T. cruzi* ergosterol biosynthesis, which is fundamental for parasite growth and survival has also shown potent *in vitro* and *in vivo* activity against *T. cruzi* (Ferraz *et al*. 2007, Olivieri *et al*. 2010, de Figueiredo Diniz *et al*. 2010, Veiga-Santos *et al*. 2012, de Figueiredo Diniz *et al*. 2013, Moraes *et al*. 2014). However, a recent study that performed a clinical trial of posaconazole in 78 adults with chronic *T. cruzi* infections showed a very poor efficacy of this drug, and a larger percentage of treatment failures when compared with benznidazole treated patients (Molina *et al*. 2014). All these drugs are far from satisfactory due to the systemic toxicity and adverse and variable side effects that they produce (Coura *et al*. 2002, Castro *et al*. 2006). Additionally, the implementation of several drug discovery programs in the past years has enabled the discovery of promising compounds with less toxicity to combat *T. cruzi* infections such as fexinidazole, and fenarimol derivatives (Keenan *et al*. 2012, Hargrove *et al*. 2013, Keenan *et al*. 2013, Moraes *et al*. 2014).

Other drugs that have been shown to present activity against trypanosomes, and that are currently used to combat infections with *T. brucei* and *Leishmania* are melarsoprol and miltefosine. Miltefosine is an alkylphosphocholine originally developed as an

anticancer drug, but it has also shown activity against different species of *Trypanosoma*, *Leishmania*, *Entamoeba histolytica*, and *Trichomonas vaginalis* (Dorlo *et al*. 2012). The anti-leishmanial activity of miltefosine was initially discovered in the mid 1980s, and the subsequent demonstration of its efficacy in several experimental models led in the mid-1990s to clinical trials and its use to combat infections with *Leishmania* (Croft *et al*. 2006). The mechanism of action of miltefosine is not clear. It has been suggested that it acts through the inhibition of phosphatidylcholine biosynthesis and sphingomyelin biosynthesis, which in turn trigger programmed apoptosis-like cell death (Paris *et al*. 2004, Verma *et al*. 2004, Marinho *et al*. 2011). However, another potential proposed target is the inhibition of cytochrome-c oxidases (Luque-Ortega *et al*. 2007).

Drugs to treat sleeping sickness are old, scarce, and highly toxic (Torreele *et al*. 2010). Pentamidine and suramin were both developed more than half a century ago and are still currently used to treat the early stage of infection with *T. b. gambiense* and *T. b. rhodesiense*. However, severe side effects have often been reported, including anaphylactic shocks, severe cutaneous reactions, neurotoxic signs, and cases of renal failure (Steverding. 2010). Melarsoprol is used to treat second-stage sleeping sickness caused by both *T. b. gambiense* and *T. b. rhodesiense* (Steverding. 2010). It is an organo-arsenical compound that is highly toxic (Travis. 1991) with many adverse side effects such as a reactive encephalopathy caused by the presence of trypanosomes in the central nervous system (CNS) (Baker *et al*. 2013). It has been suggested that the inflammation in the CNS is triggered by the lysis of trypanosomes (Pepin *et al*. 1994).

Some drugs developed against certain *Trypanosoma* species have been shown to be active against others. This is the case of miltefosine, which is used against *Leishmania*,

but has also been shown to have activity against *T. cruzi* and *T. b. rhodesiense in vivo* and *in vitro,* although the efficacy against these species is lower than against *Leishmania* (Croft *et al*. 1996, Konstantinov *et al*. 1997). Furthermore, some drugs currently used against pathogenic trypanosomes have been screened *in vitro* and *in vivo* against non-pathogenic trypanosomes that are known to cause occasional pathological effects in humans and/or animals. Nifurtimox and melarsoprol for example, were shown to have activity against *T. lewisi in vitro* and *in vivo* in experimentally infected rats (Dethoua *et al*. 2013), and melarsoprol was used successfully in human infections with *T. lewisi* (Howie *et al*. 2006, Verma *et al*. 2011).

Differences at the phenotypic level in susceptibility or resistance to drugs have been reported between different strains of *T. cruzi in vitro* and *in vivo*. Differences in the susceptibility to benznidazole were observed within different *T. cruzi* strains (different discrete typing units or DTU's) *in vitro* (Luna *et al*. 2009), and *in vivo* in experimentally infected mice (Teston *et al*. 2013). The susceptibility or resistance to benznidazole and nifurtimox has been compared with genotypic characteristics using different *T. cruzi* strains as well (Murta *et al*. 1998). Roellig *et al*. (2013) suggested that differences in the sequences of some genes within and between the different DTU's of *T. cruzi* might result in phenotypic differences affecting drug susceptibility.

With the advent of complete genome sequences for the two pathogens, *T. cruzi*, and *T. brucei*, and the sequences of genes of interest in drug development processes of other trypanosomes, it is hoped that new potential drug targets will be identified, and as a consequence, new potent drugs with better efficacy and less toxicity will be discovered.

1.9 Thesis Aims

The overall aim of this research project was to investigate the genotypic and phenotypic diversity of *Trypanosoma* spp. infecting Western Australian marsupials, and to examine their potential virulence and pathogenicity with particular emphasis on those trypanosomes associated with the critically endangered marsupial, the woylie or brush-tailed bettong (*Bettongia penicillata*).

More specifically, this project aimed to:

- Investigate the genetic diversity and phylogenetic relationships of trypanosomes present within Western Australian marsupials.
- Develop sensitive species-specific polymerase chain reaction protocols (PCR's) to discriminate between the different species of *Trypanosoma*.
- Investigate the capacity of trypanosomes to infect different tissue cells in natural infected animals, and *in vitro* using different cell lines and trypanosomes isolated in culture.
- Describe the morphology and ultrastructure of trypanosomes isolated in culture.
- Examine the potential pathogenicity of trypanosomes in the woylie.
- Determine trypanosomes susceptibility to different drugs *in vitro.*
- Investigate trypanosomes kinetoplast DNA organisation.

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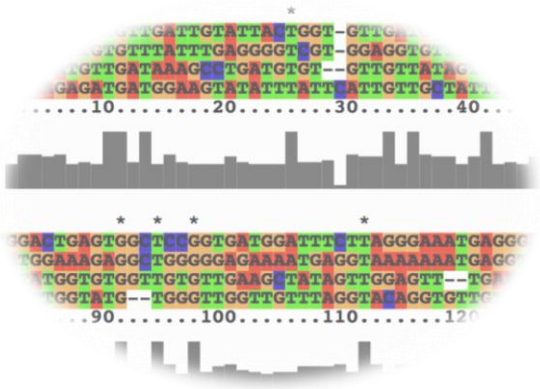
Chapter 2

Genetic diversity, phylogenetic relationships and tissue distribution of trypanosomes infecting Western Australian marsupials



2.1 Abstract

Trypanosomes are blood protozoan parasites that infect humans, livestock and wildlife. While much is known about the genetic diversity and life cycle of trypanosomes that affect humans and livestock, less is known about trypanosomes in wildlife. This study describes the genetic diversity and tissue tropism of trypanosomes naturally infecting Western Australian marsupials. Blood samples collected from 554 live-animals and 250 tissue samples extracted from 50 carcasses of sick-euthanised or road-killed animals, belonging to nine species of marsupials, were screened for the presence of trypanosomes using a PCR of the 18S rDNA gene. PCR results showed all species harboured trypanosomes and revealed a rate of infection of 67% in blood and 60% in tissues. Inferred phylogenetic trees using 18S rDNA and glycosomal glyceraldehyde phosphate dehydrogenase (g*GAPDH*) sequences showed the presence of eight genotypes that clustered into three clades: a clade including *T. copemani* (Clade A), a new clade closely related to *T. gilletti* (Clade B – described as *T. vegrandis* clade in Chapter 4), and a clade including *T.* sp H25 previously isolated from an eastern grey kangaroo (Clade C). Clade-specific primers were constructed based on the aggregation of the different genotypes in the phylogenetic tree obtained from the 18S rDNA sequences (1,410 bp). PCR using clade-specific primers showed a higher prevalence of infection with trypanosomes from Clade A followed by Clade B and Clade C in both blood and tissues. The results obtained provided evidence of the wide genetic diversity and lack of host specificity of trypanosomes within Australian marsupials, and demonstrates for the first time the capacity of Australian trypanosomes to migrate to different tissues in the vertebrate host.

2.2 Background

Different species of *Trypanosoma* have been described infecting humans, livestock and wildlife. *T. cruzi* and *T. brucei*, trypanosomes from America and Africa respectively, cause fatal diseases by affecting vital organs in the vertebrate host. However, differences in the biological behaviour in the host have been demonstrated between species. Although trypanosomes are obligate hematozoan parasites, some species are capable of migrating to different organs in the vertebrate host. *T. cruzi* for example, migrates to several tissues and once there is able to infect and multiply within host cells (De Souza *et al*. 2010). In contrast, *T. brucei* replication occurs extracellularly in the bloodstream. However, this species has also been found in blood vessels and capillaries of some organs such as the kidney and in the brain (Frevert *et al*. 2012).

While much is known of trypanosomes that affect humans, a great deal less is known of those associated with wildlife (Averis *et al*. 2009). Several *Trypanosoma* species have been described from wildlife. *T. cruzi* for example, has been reported in the blood of more than 150 wild mammalian species in the Americas (Rozas *et al*. 2007) and many other species of trypanosomes have been found in the blood of birds, fish, reptiles, rodents, marsupials, and bats (Hoare. 1972). Whilst most of these studies have focused on the description of trypanosomes from blood, a limited number have involved the search for trypanosomes in different host tissues. To date, only a few species of trypanosomes that affect wildlife have been shown to be capable of migrating to and infecting different tissue cells during natural infections. An early study detected uncharacterised "*T. cruzi* like" trypanosomes from primates (*Nycticebus coucang*) from Malaysia inside heart muscle cells (Kuntz *et al*. 1970). *T. dionissi* and *T. erneyi*, both bat trypanosomes, have also been shown to infect host tissue cells (Baker *et al*. 1972,

Lima *et al*. 2012, Lima *et al*. 2013). In contrast, the life cycle of the majority of wildlife trypanosomes described so far does not involve tissue infection, and many studies have failed to demonstrate intracellular stages in host tissues. New species of *Trypanosoma* were found in the blood of caimans (*Caiman crocodilus* and *Caiman yacare* respectively) from Brazil. However, when caiman tissues were examined, non-intracellular stages of the parasite were found, but lung and kidney imprints from two caimans revealed scarce trypomastigotes distinct from those detected in peripheral blood samples (Viola *et al*. 2009, Marcili *et al*. 2013). In addition, no intracellular forms were seen in different tissues collected from rice-field frogs (*Hoplobatrachus rugulosus*) naturally infected with *T. rotatorium* and *T. chattoni* from Thailand (Sailasuta *et al*. 2011).

Within Australia, numerous species and genotypes of *Trypanosoma* have been described naturally infecting a wide range of native marsupials. These include *T. thylacis* in northern brown bandicoots (*Isoodon macrourus*), *T. irwini* and *T. gilletti* in koalas (*Phascolarctos cinereus*) (McInnes *et al*. 2009, McInnes *et al*. 2011b), and *T. copemani* in quokkas (*Setonix brachyurus*), Gilbert's potoroo (*Potorous gilbertii*) (Austen *et al*. 2009) and koalas (McInnes *et al*. 2011b). Different uncharacterised species are also seen in the blood of the woylie (*Bettongia penicillata*), common wombat (*Vombatus ursinus*), eastern grey kangaroo (*Macropus giganteus*), swamp wallaby (*Wallabia bicolor*) brush tailed possum (*Trichosurus vulpecula*), chuditch (*Dasyurus geoffroii*), golden bandicoot (*Isoodon auratus*), southern brown bandicoot (*Isoodon obesulus*) and burrowing bettong (*Bettongia lesueur*) (Bettiol *et al*. 1998, Noyes *et al*. 1999, Hamilton *et al*. 2005, Smith *et al*. 2008, Averis *et al*. 2009, Paparini

et al. 2011). However, the capacity of Australian trypanosomes to migrate to different tissues in the vertebrate host has never been studied.

Phylogenetic analysis has revealed that most of the Australian trypanosomes fall into several distinct clades. Some of them are closely related to trypanosomes from outside Australia. *T. sp* H25, *T. binneyi, T. sp* ABF and *T. sp* AAT, for example, have been shown to be closely related to trypanosomes from America, Portugal, Malaysia, and Thailand respectively, suggesting that Australian trypanosomes may have divergent evolutionary origins (Hamilton *et al*. 2005). However, several new species and genotypes have been described since 2008, and the majority clustered with species/genotypes within Australia (Smith *et al*. 2008, Averis *et al*. 2009, Austen *et al*. 2009, McInnes *et al*. 2011b, Paparini *et al*. 2011).

Despite the identification of this large list of Australian trypanosomes and their marsupial hosts, there are many unanswered questions related to their host specificity and biological behaviour in the vertebrate host. This chapter aims to investigate the genetic diversity and phylogenetic relationships of trypanosomes that are prevalent in Western Australian marsupials, as well as to determine their capability to migrate to different tissues in the vertebrate host during natural infections.

2.3 Materials and methods

2.3.1 Sample collection

Tissue and blood samples were collected from nine species of marsupials at different locations throughout Western Australia (WA) as part of the WA Department of Parks

and Wildlife (DPaW) fauna research and monitoring program (Figure 1). Heparinised peripheral blood samples were taken from a total of five hundred and fifty four trapped and released marsupials during ongoing trapping sessions by DPaW (Table 1). Tissue samples were collected from sick-euthanised animals that were presented to Perth Zoo Veterinary Department for treatment and from dead (accidently killed on roads) animals sent to Murdoch University for necropsy. Sick animals were euthanised due to very poor body condition, marked lethargy and poor prognosis for return to the wild. A total of two hundred and fifty tissue samples were collected from fifty carcasses (Table 1). Tissues were collected from at least two of the following organs from each animal: spleen, liver, lung, heart, kidney, brain, oesophagus, tongue, skeletal muscle and bone marrow. Wildlife sampling was carried out under Murdoch University animal ethics approval permit numbers NS1182-06, W2172-08 and W2350-10, and DPaW animal ethics approval permit number DECAEC/52/2009. All tissue samples were extensively washed with phosphate buffered saline (PBS) and stored in 100% ethanol for DNA isolation.

Table 1. Blood and tissue samples collected from different species of Western Australian marsupials

Marsupial species	Blood samples	Carcasses	Tissue samples
Woylie *(Bettongia penicillata)*	494	27	154
Quenda (*Isoodon obesulus*)	2	2	11
Quokka *(Setonix brachyurus)*	-	3	7
Tammar wallaby *(Macropus eugenii)*	-	3	7
Banded hare wallaby *(Lagostrophus fasciatus)*	1	-	-
Boodie *(Bettongia lesueur)*	7	1	3
Chuditch *(Dasyurus geoffroii)*	2	4	30
Common brush tailed possum *(Trichosurus vulpecula)*	7	-	-
Western grey kangaroo *(Macropus fuliginosus)*	41	9	38
Total	**554**	**50**	**250**

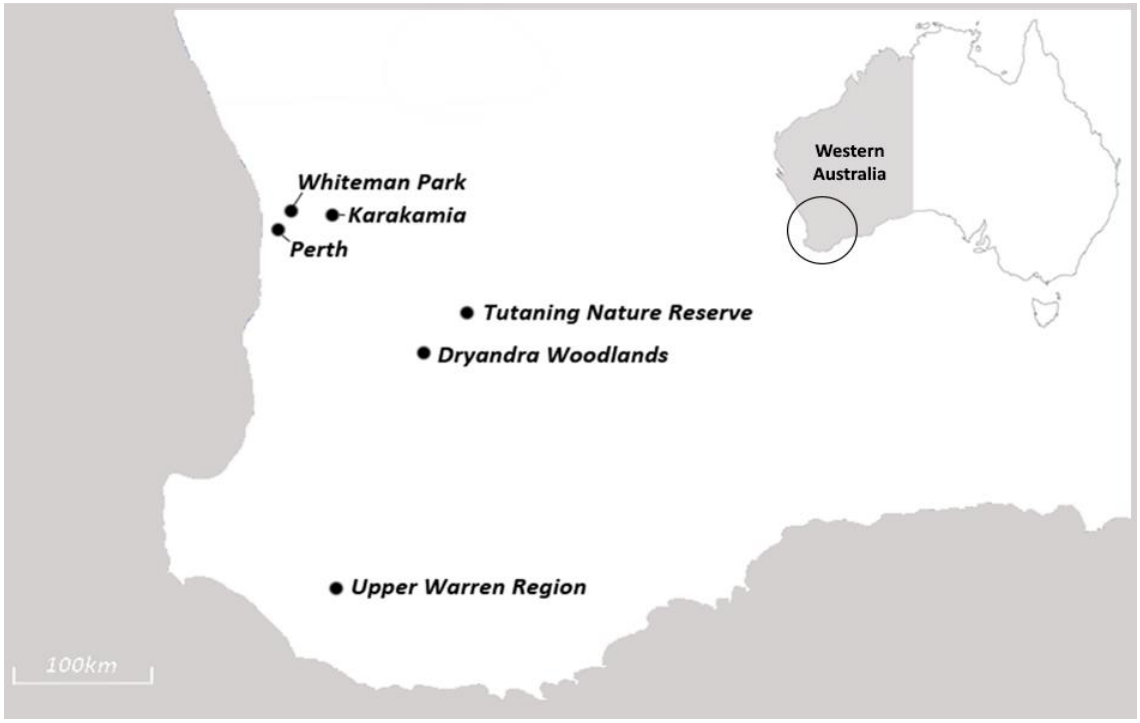


Figure 1. Geographical origin of the different species of marsupials trapped in this study

2.3.2 PCR amplification and sequencing of the 18S rDNA and gGAPDH loci

Genomic DNA from blood and tissues was obtained using the QIAamp blood and tissue DNA MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A nested PCR that targets a variable region of the 18S rDNA locus using generic primers that have the potential to recognise different species of trypanosomatids was performed as described previously with some modifications (Maslov *et al*. 1996, McInnes *et al*. 2011b). An initial amplification using the external primers SLF and S762R, which amplify a fragment of approximately 1.9 Kb was performed in a PT100 thermocycler (MJ Research). Then, two secondary nested PCRs were used to amplify the first 900 bp (Primers S823F and S662R) and the last 900 bp (Primers S825F and SLIR) fragments, which overlap in about 100 bp. Primer sequences, $MgCl_2$ concentrations and annealing temperatures are shown in Table 2. PCR reactions for all

primers were performed in a total volume of 25μL containing 0.2 units of Taq DNA Polymerase, 200 μM of dNTPs, 0.8 μM of each primer and 1μL of DNA template and consisted of a pre-PCR step at 94° C for 5 min, 50° C for 2 min and primer extension at 72° C for 4 min, followed by 35 cycles of 30 sec at 94° C, 30 sec at the annealing temperature in Table 2, 140 sec at 72°C and a final extension step at 72°C for 7 min.

Positive samples with 18S rDNA PCR were screened with a second PCR of the *gGAPDH* locus using modified hemi-nested reactions (McInnes *et al*. 2011b). Both PCR reactions were performed in a total volume of 25μL containing 0.2 units of Taq DNA Polymerase, 200 μM of dNTPs, 0.8 μM of each primer and 1μL of DNA template and consisted of a pre-PCR step at 94° C for 5 min, 50° C for 2 min and primer extension at 72° C for 4 min, followed by 35 cycles of 30 sec at 94° C, 30 sec at the annealing temperature shown in Table 2, 140 sec at 72°C and a final extension step at 72°C for 7 min. PCR products were run on a 1.5% agarose gel stained with SYBR safe (Invitrogen, USA), and visualised with a dark reader trans-illuminator (Clare Chemical Research, USA).

To investigate the genetic diversity of trypanosomes infecting Western Australian marsupials, two hundred positive PCR products from blood and tissue and twenty four PCR products from trypanosomes grown in culture (see Chapter 4) were chosen, purified using Agencourt AMPure PCR Purification system (manufacturer's instructions) and sequenced (18S rDNA: ~1,5kb and *gGAPDH*: ~810bp) using an ABI Prism^{TM} Terminator Cycle Sequencing kit (Applied Bio-systems, California, USA) on an Applied Bio-system 3730 DNA Analyzer. Chromatograms, derived from18S rDNA sequences (fragment 1 and 2), were used for contiguous assembly using the DNA

sequence analysis program Sequencher version 5.0.

Table 2. 18S rDNA and g*GAPDH* PCR primer sequences and PCR conditions

PCR	Primer	Primer sequence	$MgCl_2$	Annealing temperature	Band size
18S rDNA External	SLF	GCTTGTTTCAAGGACTTAGC	2.0 mM	55	~1.8 Kb
	S762R	GACTTTTGCTTCCTCTAATG			
18S rDNA Internal-fragm1	S823F	CGAACAACTGCCCTATCAGC	2.0 mM	56	~ 900bp
	S662R	GACTACAATGGTCTCTAATC			
18S rDNA Internal-fragm2	S825F	ACCGTTTCGGCTTTTGTTGG	1.5 mM	57	~ 950bp
	SLIR	ACATTGTAGTGCGCGTGTC			
g*GAPDH* External	GAPDF	CTYMTCGGNAMKGAGATYGAYG	2.0 mM	59	~ 900bp
	GAPDR	GRTKSGARTADCCCCACTCG			
g*GAPDH* Internal	GAPDF	CTYMTCGGNAMKGAGATYGAYG	2.0 mM	55	~ 880bp
	G4a	GTTYTGCAGSGTCGCCTTGG			

2.3.3 DNA sequence alignments and phylogenetic inferences

18S rDNA and g*GAPDH* sequences obtained from blood, tissue and cultured trypanosomes, were manually refined using BioEdit 7.0.9.0 (Hall. 1999), and then aligned using MUSCLE (Edgar. 2004). Three different alignments were created for phylogenetic inference. First, 1,410 bp sequences of 18S rDNA from trypanosomes obtained in the current study were aligned with 31 *Trypanosoma* spp. sequences representing all known trypanosome clades, and five other trypanosomatid sequences for use as outgroups obtained from GenBank. Secondly, truncated (786 bp) sequences of 18S rDNA were aligned with those previously published from different Western Australian marsupials (Smith *et al*. 2008, Averis *et al*. 2009, Austen *et al*. 2009). Finally, 810 bp g*GAPDH* sequences obtained in the current study were aligned with 26 *Trypanosoma* spp. sequences representing all major trypanosome clades, and five other trypanosomatid sequences for use as outgroups. All *Trypanosoma* spp. and outgroup sequences obtained from GenBank are shown in Table 3. JModelTest 2.1.1 was used to find the most appropriate nucleotide substitution model for ML and Bayesian analyses

(Posada. 2008). The models of nucleotide substitution chosen were: TIM3ef+I+G (equal-frequencies transition model plus gamma) for the first alignment and GTR+I+G (general time reversible gamma proportion of invariant sites) for the other two alignments. The posterior probability distribution was estimated using The Markov chain Monte Carlo, which was run for 10,000,000 generations, until the mean standard deviation of split frequencies was lower than 0.01. Trees were sampled every 100th generations and the first 2,500 trees (first 250,000 generations), which usually present very low likelihood values, were discarded (burn-in phase) as shown in Figure 2.

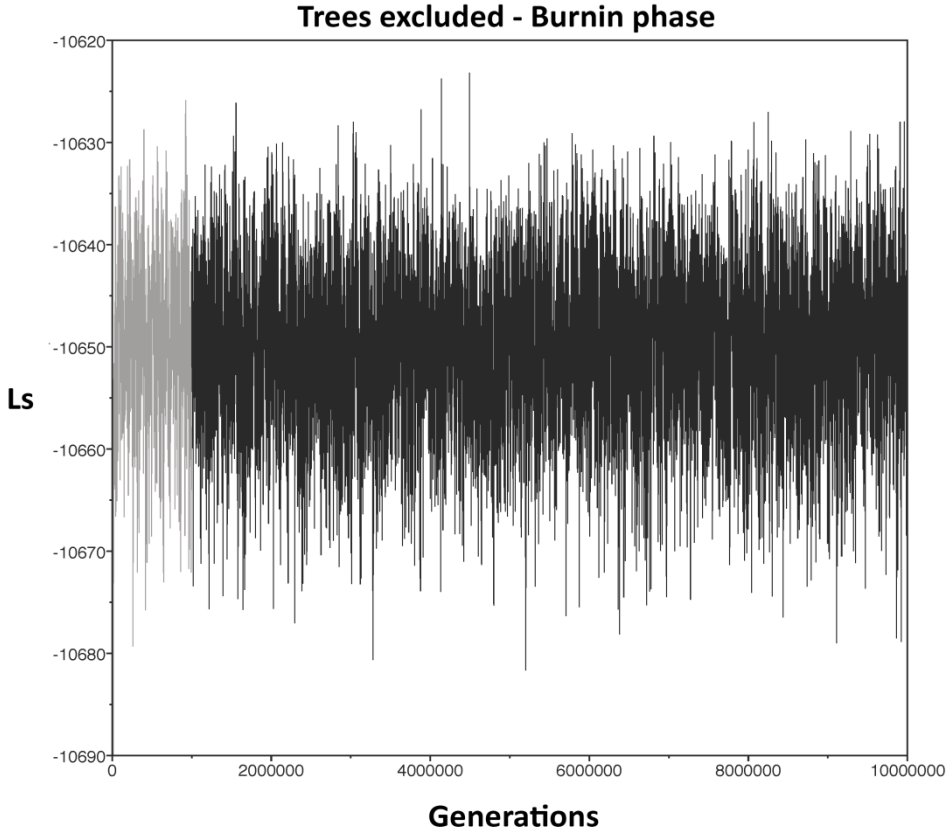


Figure 2. Burn-in value predicted for the 18S rDNA phylogenetic tree

Table 3. GenBank accession number of the sequences used in the phylogenetic analysis

Isolates	**18S rDNA Accession number**	***gGAPDH* Accession number**
T. rotatorium	AJ009161	AJ620256
AAT (Currawong)	AJ620557	
AAI	AJ620559	-
KG1 (Tick)	-	FJ649492
T. mega	AJ009157	AJ620253
T. binneyi	AJ132351	AJ620266
T. granulosum	AJ620551	-
T. avium Rook	U39578	-
T. theileri	AJ009164	AJ620282
T. Cyclops	AJ131958	
ABF (Wallaby)	AJ620564	AJ620278
H25 (Kangaroo)	AJ009168	AJ620276
H26	AJ009169	-
T. dionisii	AJ009151	FN599054
T. cruzi marinkellei	AJ009150	AJ620270
T. cruzi COLOMBIANA	AF239980	-
T. cruzi SLU31 clone2	AY785586	-
T. cruzi VINCH89	AJ009149	AJ620269
T. rangeli	AJ009160	AF053742
T. vespertilionis	AJ009166	AJ620283
T. conorhini	AJ012411	AJ620267
T. pestanai	AJ009159	AJ620275
AAP	AJ620558	AJ620277
T. lewisi	AJ009156	AJ620272
T. microti	AJ009158	AJ620273
T. gilletti Lanie	GU966589	GU966587
T. copemani Charlton (Koala)	GU966588	GU966585
T. copemani Mika (Koala)	-	GU966585
T. copemani Harrison (Koala)	-	GU966586
T. irwini	FJ649479	FJ649485
T. bennetti	AJ223562	FJ649486
T. copemani Q3 (Quokka)	EU571232	-
T. copemani Q10 (Quokka)	EU571234	-
T. copemani GP94 (Gilbert's potoroo)	EU571231	-
T. copemani GP63 (Gilbert's potoroo)	EU571233	-
TRY1 (Woylie)	EU518939	-
TRY2 (Woylie)	EU518940	-
WYA1 (Woylie)	FJ823116	-
WYA2 (Woylie)	FJ823121	-
CHA1 (Chuditch)	FJ823120	-
T. sp AP2011 isolate27 clone4	JN315394	-
T. sp AP2011 isolate4 clone6	JN315392	-
T. sp AP2011 isolate28 clone11	JN315387	-
T. sp AP2011 isolate 15	-	JN315395
T. sp AP2011 isolate 17	-	JN315396
Leptomonas sp.	AF153043	AF339451
Phytomonas serpens	U39577	EU084892
H. muscarum	L18872	DQ092548
H. samuelpessoai	U01016	AF047494
H. megaseliae	U01014	DQ092547

2.3.4 Trypanosoma clade-specific PCR

Trypanosoma clade-specific primers were constructed based on the aggregation of the different genotypes (eight genotypes) in the phylogenetic tree obtained from the 1,410 bp 18S rDNA sequences (three clades). Specific primers that amplify *T. copemani* and could recognise genotype 1 (G1) and genotype 2 (G2) (both in Clade A) were used as described previously (McInnes *et al*. 2011b). Two sets of specific primers that amplify the other major genotype groups, genotypes 3-7 (G3 to G7) (Clade B) and genotype 8 (G8) (Clade C) were constructed as shown in Table 4. PCR reactions for all blood and tissue samples were performed in a total volume of 25μL containing 0.2 units of Taq DNA Polymerase, 200 μM of dNTPs, 0.8 μM of each primer and 1μL of DNA template. Amplification was performed in a PT100 thermocycler (MJ Research) and consisted of a pre-PCR step at 94°C for 5 min, 50°C for 2 min and primer extension at 72°C for 4 min followed by 35 cycles of 30 sec at 94°C, 30 sec at the annealing temperature described in Table 4 for each clade, 50 sec at 72°C and a final extension step at 72°C for 7 min. PCR products were run on a 1.5% agarose gel stained with SYBR safe (Invitrogen, USA), and visualised with a dark reader trans-illuminator (Clare Chemical Research, USA). Prevalence of infection with trypanosomes was expressed as the percentage of samples found positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa *et al*. 2000).

Table 4. Clade B and Clade C - specific PCR conditions and primer sequences

PCR	Primer	Primer sequence	$MgCl_2$ Concentration	Annealing temperature	Band Size
Clade C External	H25EF	GCCGACAGTGCATTTTGT	1.0 mM	60	~ 750bp
	H25ER	GAGCGAGATGAACTCGACC			
Clade C Internal	H25IF	TTTGAGGCGCAATGGTTTAG	1.0 mM	60	~ 400bp
	H25IR	CGAGTTGAGGGAAGGTGGC			
Clade B External	TVEF	GGGGTCCTTTTATTTTATTTG	1.5 mM	58	~ 750bp
	TVER	TAATTTATTGGCCAGACAAA			
Clade B Internal	TVIF	GACCAAAAACGTGCACGTG	1.0 mM	58	~ 350bp
	TVIR	AAATCGTCTCCGCTTTAAC			

2.4 Results

2.4.1 Rate of infection in blood and tissue revealed by PCR using generic primers

Blood samples from 554 trapped and released marsupials and 250 tissue samples extracted from 50 carcasses of dead or euthanised marsupials were screened for the presence of trypanosomes using generic primers for 18S rDNA. At least one individual of each marsupial species examined was positive for *Trypanosoma* infection either in blood or tissue. The rate of infection over all host species was 67% in blood of trapped and released animals and 60% in carcasses of dead animals, where at least one tissue sample was positive (Table 5).

Table 5. Overall prevalence of *Trypanosoma* infection in blood and tissues of different species of Western Australian marsupials

Marsupial species	Blood samples (%, 95% CI)	Carcasses (%, 95%CI)	Tissue samples (%, 95% CI)
Woylie *(Bettongia penicillata)*	335/494 (68, 63-71)	18/27 (67, 46-81)	67/154 (43, 35-51)
Quenda (*Isoodon obesulus*)	0/2	2/2	8/11
Quokka *(Setonix brachyurus)*	-	1/3	2/7
Tammar wallaby *(Macropus eugenii)*	-	1/3	3/7
Banded hare wallaby *(Lagostrophus fasciatus)*	1/1	-	-
Boodie *(Bettongia lesueur)*	1/7	0/1	0/3
Chuditch *(Dasyurus geoffroii)*	1/2	4/4	17/30
Common brush tailed possum *(Trichosurus vulpecula)*	3/7	-	-
Western grey kangaroo *(Macropus fuliginosus)*	29/41	4/9	9/38
Total	**370/554 (67, 63-71)**	**30/50 (60, 48-75)**	**106/250 (42, 36-48)**

* The rate of infection is given by: number of *Trypanosoma*-positive samples or carcasses/total number of samples or carcasses. 95% CI (95% confidence interval)

2.4.2 Phylogenetic analysis and evolutionary divergence

Eight different genotypes (G1 to G8) from three distinct clades were found at the 18S rDNA locus among the 200 blood and tissue samples chosen for sequencing (Table 6).

Table 6. GenBank accession number of the new reported sequences and origin of the isolates.

GenBank Accession number 18S rDNA	GenBank Accession number g*GAPDH*	*Trypanosoma* Genotype	*Trypanosoma* Clade	Hosts
KC753530	KC812982	Genotype 1 (G1)	Clade A	Woylie, common brush-tailed possum
KC753531	KC812983	Genotype 2 (G2)	Clade A	Woylie, quokka, chuditch, quenda
KC753532	KC812984	Genotype 3 (G3)	Clade B	Woylie, Western grey kangaroo
KC753533	KC812985	Genotype 4 (G4)	Clade B	Woylie, quenda
KC753534	KC812986	Genotype 5 (G5)	Clade B	Woylie, tammar wallaby
KC753535	-	Genotype 6 (G6)	Clade B	Woylie, chuditch
KC753536	KC812987	Genotype 7 (G7)	Clade B	Woylie
KC753537	KC812988	Genotype 8 (G8)	Clade C	Woylie, banded hare wallaby, boodie

Very similar phylogenetic relationships were found using both Bayesian and ML analyses between these eight genotypes and the 31 trypanosome sequences obtained from GenBank (only the results from the Bayesian analysis are shown). The eight genotypes found in this study were grouped into three distinct clades (Figure 3A). The first two genotypes (G1 and G2) clustered within a clade including *T. copemani* (Clade A)*;* G1 showed a 99% and 98% similarity to *T. copemani* previously described from wombats and koalas respectively (Noyes *et al*. 1999, McInnes *et al*. 2011b), and G2 was closely related to G1 but differed in 15 nucleotides among the 1,410 bp of sequence. Five genotypes (G3 to G7) clustered altogether in a new clade (Clade B) described as *T. vegrandis* clade in Chapter 4 (Thompson *et al*. 2013). All Clade B genotypes clustered with *T. sp* AP2011-isolate 27, *T. sp* AP2011-isolate 28 and *T. sp* AP2011-isolate 4, previously described from woylies (Paparini *et al*. 2011). In all analyses, the closest relative of this clade among previously described trypanosome species was *T. gilletti* from koalas, although the nucleotide distances between *T. gilletti* and the genotypes within this clade were considerably large (Table 7). The last genotype found (G8) presented 99% similarity with a trypanosome previously isolated from an eastern grey kangaroo from Victoria - *T. sp* H25 (Noyes *et al*. 1999). Genotypes within this clade (Clade C) were more closely related to trypanosomes from outside Australia such as *T. cruzi* and *T. rangeli* from South America and were previously positioned in a monophyletic assemblage designated as the "*T. cruzi* clade" (Hamilton *et al*. 2012). Phylogenetic analysis of the eight new genotypes found in this study and truncated genotypes previously published from Western Australian marsupials demonstrated close relationships between them. Trypanosome isolates from a chuditch (CHA1) and woylies (TRY1, TRY2, WYA1, WYA2, *T.* sp AP2011), clustered within Clade B, while *T. copemani* isolates from quokkas (Q3 and Q10), koalas (Charlton) and Gilbert's

potoroos (GP63 and GP94) and isolates from wombats (AAP, H26, AAI) clustered together with G1 and G2 in Clade A (Figure 3B). However, all of them were more closely related to G1 than to G2. Results showed G1 trypanosomes shared a more recent common ancestor with *T. copemani* from quokkas, koalas, Gilbert's potoroos and wombats than to G2 trypanosomes. Additional support for the phylogenetic positioning of the new sequences was provided by the phylogenies derived from the g*GAPDH* sequences (Figure 4), which showed the same topology as the 18S rDNA derived tree, although only seven genotypes were included due to the lack of PCR amplification of G6. With a Bayesian support value of 1 (100%), phylogenies based on both 18S rDNA and gGAPDH genes showed that trypanosomes from Clade A, Clade B, and *T. gilletti* (all trypanosomes from Australian marsupials) shared a common ancestor with *T. pestanai* isolated from badgers in Europe (Lizundia *et al*. 2011).

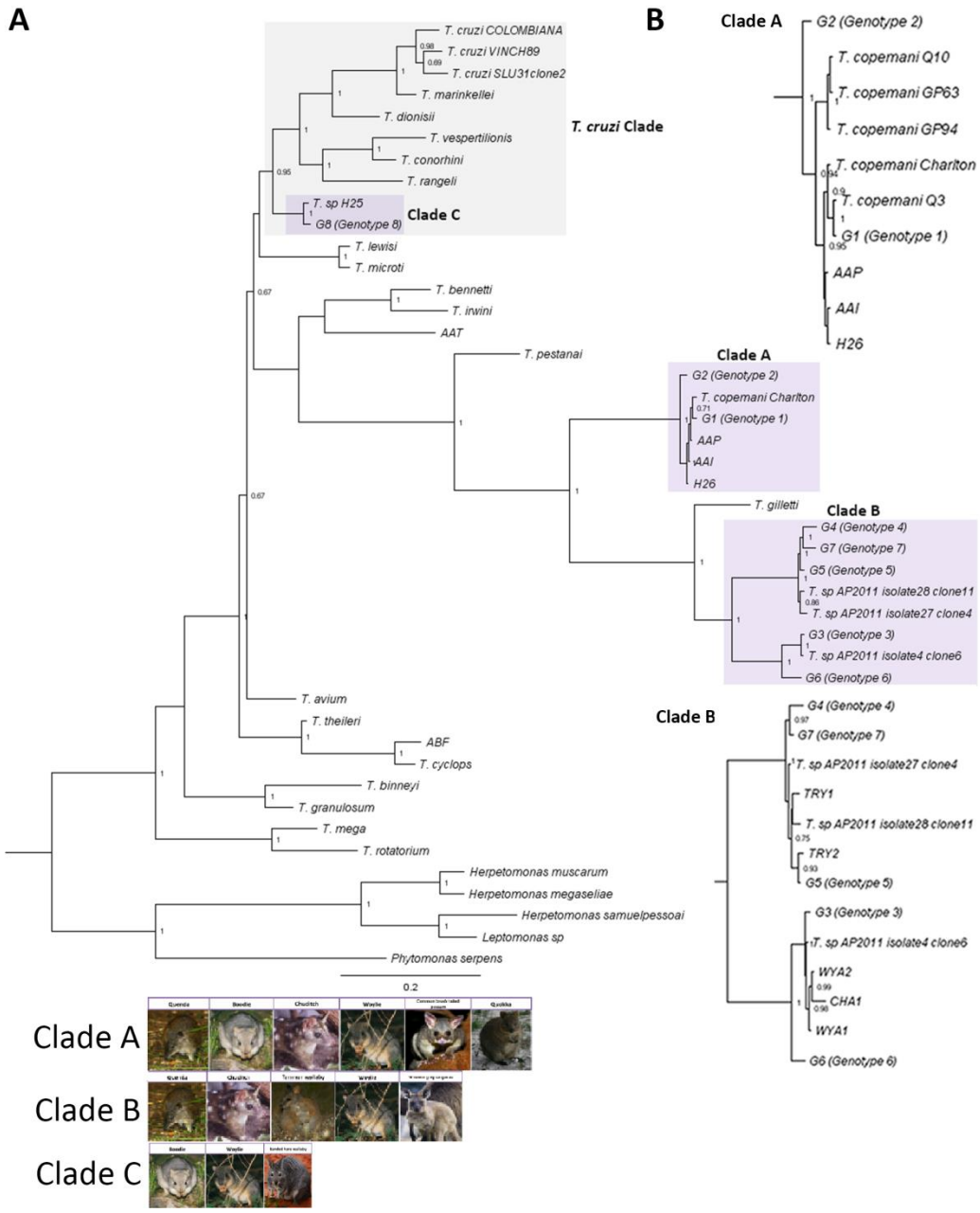


Figure 3. Phylogenetic analysis of the relationships between Australian trypanosomes based on 18S rDNA sequences. Phylogenetic trees were constructed by the Bayesian method. A: phylogenetic position of longer 18S rDNA sequences (~1,410 bp); B: phylogenetic position of shorter 18S rDNA sequences (786 bp) CHA1, TRY1, TRY2, WYA1, WYA2, BDA1, Q3, Q10, GP63 and GP94. Trees were rooted with five sequences as outgroups. Bootstrap values from Bayesian posterior probabilities are shown at nodes. Bar: 0.2 substitutions per site.

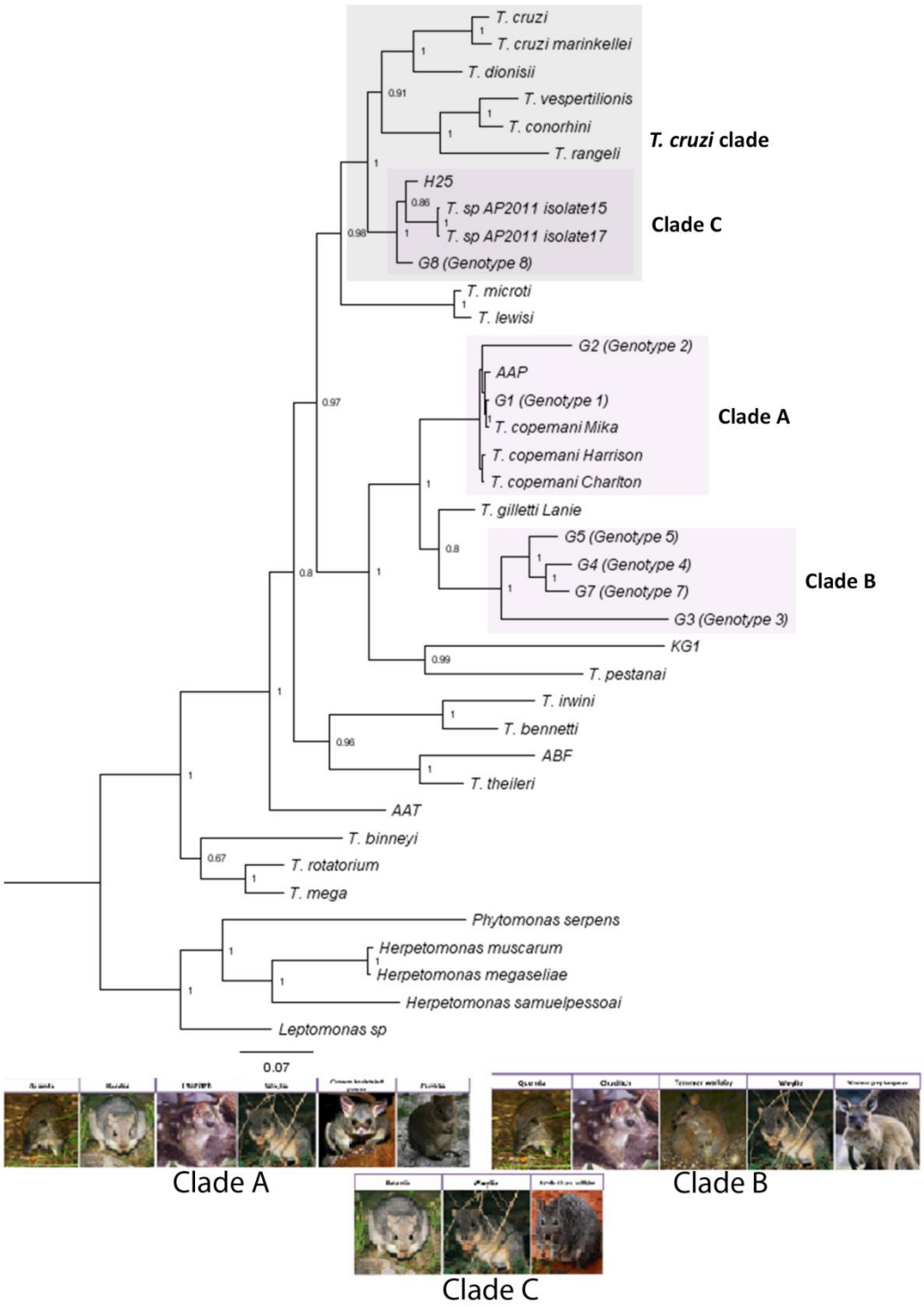


Figure 4. Phylogenetic relationships of the new trypanosome isolates from Western Australian marsupials based on *gGAPDH* sequences (~810 bp) using Mr Bayes. The tree was rooted with five sequences as outgroups. Bayesian posterior probabilities are shown at nodes. Bar: 0.07 substitutions per site.

Results of the evolutionary divergence from all genotypes confirmed the relationships obtained in the phylogenies. The evolutionary divergence between trypanosomes previously described in wombats (*T. sp* AAI, AAP and H26) and G1 was lower (0.002) than that one obtained between these wombat trypanosomes and G2 (0.010). Moreover, the sequence divergence between G2 and the closest relative *T. gilletti* was higher than the one observed between *T. gilletti* and G1 indicating that although both G1 and G2 belong to Clade A, G1 is more closely related to *T. gilletti* than G2 (Table 7). Despite *T. pestanai* sharing a recent common ancestor with trypanosomes from Clade A and Clade B as shown in the phylogenies (Figure 3 and 4), the genetic distance between *T. pestanai* and Clade C was lower (0.068) when compared with the genetic distances with Clade A and Clade B trypanosomes (0.082-0.115).

Trypanosoma		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
G1	A		0.003	0.008	0.008	0.009	0.008	0.009	0.008	0.009	0.001	0.009	0.008	0.009	0.007	0.009	0.008
G2	B	0.012		0.008	0.008	0.009	0.008	0.009	0.008	0.009	0.003	0.009	0.008	0.009	0.007	0.009	0.008
G3	C	0.083	0.089		0.006	0.007	0.003	0.007	0.009	0.009	0.008	0.009	0.005	0.009	0.008	0.009	0.009
G4	D	0.104	0.107	0.052		0.004	0.006	0.003	0.009	0.009	0.008	0.009	0.006	0.009	0.008	0.009	0.009
G5	E	0.114	0.116	0.065	0.018		0.007	0.004	0.009	0.009	0.009	0.010	0.007	0.009	0.008	0.010	0.009
G6	F	0.087	0.094	0.013	0.056	0.069		0.007	0.009	0.009	0.008	0.009	0.006	0.009	0.008	0.009	0.009
G7	G	0.111	0.113	0.060	0.013	0.020	0.064		0.009	0.009	0.009	0.009	0.007	0.009	0.008	0.009	0.009
G8	H	0.098	0.094	0.101	0.105	0.117	0.109	0.112		0.006	0.008	0.006	0.009	0.006	0.007	0.005	0.002
T. marinkellei	I	0.114	0.114	0.118	0.129	0.138	0.132	0.136	0.047		0.009	0.004	0.009	0.006	0.008	0.007	0.005
T. sp AAI, AAP, H26	J	0.002	0.010	0.086	0.105	0.114	0.090	0.112	0.098	0.111		0.009	0.008	0.009	0.007	0.009	0.008
T. cruzi	K	0.119	0.118	0.113	0.137	0.146	0.133	0.143	0.047	0.023	0.118		0.009	0.007	0.008	0.007	0.006
T. gilletti	L	0.076	0.079	0.036	0.060	0.072	0.041	0.068	0.103	0.124	0.077	0.129		0.009	0.008	0.009	0.009
T. lewisi	M	0.096	0.094	0.103	0.118	0.130	0.112	0.126	0.038	0.055	0.096	0.059	0.112		0.007	0.007	0.006
T. pestanai	N	0.084	0.082	0.088	0.104	0.115	0.098	0.112	0.068	0.085	0.084	0.092	0.096	0.073		0.007	0.007
T. rangeli	O	0.115	0.112	0.111	0.130	0.142	0.122	0.137	0.037	0.067	0.115	0.070	0.121	0.061	0.081		0.005
T.sp H25	P	0.098	0.094	0.106	0.107	0.119	0.112	0.115	0.003	0.043	0.098	0.045	0.105	0.037	0.070	0.038	

Table 7. Estimates of evolutionary divergence between the 18S rDNA sequences from all the genotypes found in this study. Sequences from other *Trypanosoma* species are also shown. The number of base substitutions per site between sequences is shown. Standard error estimate(s) are shown above the diagonal (blue) and were obtained by a bootstrap procedure (10000 replicates). Analyses were conducted using the Kimura 2-parameter model. Genotypes 1 and 2 (Clade A), genotype 3 to 7 (Clade B), genotype 8 (Clade C).

The genetic distances of G3 and G6 with G4, G5 and G7 trypanosomes (all from Clade B), fluctuated between 0.052 and 0.064. These distances were significantly higher than distances between two closely related *Trypanosoma* species from the Americas, *T. cruzi* and *T. marinkellei* (0.023), possibly indicating that G3 and G6 trypanosomes might comprise a separate species. These results are in agreement with both 18S rDNA and g*GAPDH* trees, which showed two small sub-clades in Clade B comprising G4, G5, and G7 (first sub-clade) and G3 and G6 (second sub-clade).

2.4.3 Trypanosome clade-specific PCR

To confirm the reliability of trypanosome clade-specific primers and PCRs, DNA sequencing was carried out from 16 blood samples infected with genotypes representative of the three different clades. These sequences showed a 100% similarity with the full 18S rDNA sequences, and confirmed the high specificity of the clade-specific primers and PCRs.

Out of the 370 blood samples positive for trypanosome infection, 53% (95% CI= 49-57) were positive for Clade A trypanosomes, 32% (95% CI= 27-37) for Clade B trypanosomes, 2% (95% CI=1-3) for Clade C trypanosomes and 13% (95% CI=10-17) were mixed infections with two or more genotypes representative of different clades. From the 106 tissues infected, 47% (95% CI=38-57) were positive for Clade A, 29% (95% CI=21-39) for Clade B, 2% (95% CI=0.3-7) for Clade C, and 22% (95% CI=14-31) for mixed infections. Although PCR using clade-specific primers showed the presence of mixed infections in some of the samples, sequencing of the same samples using the 18S rDNA trypanosome generic primers revealed infections with only one genotype, thus possibly indicating that this PCR might amplify preferentially more

abundant genotypes in the sample.

Parasite genotypes were not host-specific, with most being found in at least two host species (Table 6). Whereas single infections were found in blood and different tissues of all marsupials examined, infections were only present in blood and tissues of woylies. This host species presented a total of 13% (95% CI=10-17) and 1% (95% CI=0.2-3) of dual infections with genotypes belonging to Clade A-Clade B and Clade B-Clade C respectively. Two percent of the samples (95% CI=0.8-4) presented triple infections with genotypes from the three clades. All trypanosome genotypes were present in peripheral blood. However, only G2 (Clade A), G3, G4 and G5 (Clade B) and G8 (Clade C) were found infecting host tissues, including tissues from woylies, quokkas, quendas, tammar wallabies, chuditch and western grey kangaroos. Trypanosomes within Clade A exhibited a preferential tissue distribution for skeletal muscle, tongue, oesophagus, and heart, while trypanosomes within Clade B showed predilection for skeletal muscle, heart and lung. Table 8 shows the rate of infection among all different tissues collected, including tissues infected with more than one genotype.

Table 8. Differential tissue distribution of trypanosomes within the different clades

Trypanosoma clades	Spleen	Kidney	Liver	Lung	Heart	Tongue	Skeletal Muscle	Oeso-phagus	Brain	Bone Marrow
Clade A	10% (2-26)	17% (5-37)	25% (13-43)	21% (9-37)	37% (19-58)	40% (16-68)	41% (25-59)	38% (14-68)	0%	9% (2-41)
Clade B	7% (1-22)	12% (26-32)	8% (16-21)	16% (60-31)	18% (6-38)	13% (16-40)	47% (29-65)	15% (19-45)	0%	9% (2-41)
Clade C	3% (0.1-17)	4% (0.1-21)	3% (0.1-14)	5% (0.6-18)	0%	0%	0%	0%	0%	0%
TOTAL	**20% (8-38)**	**33% (16-55)**	**37% (22-54)**	**42% (26-59)**	**56% (35-74)**	**53% (26-79)**	**88% (72-97)**	**54% (25-81)**	**0%**	**18% (2-52)**

* The rate of infection (%) in each organ is given by the number of *Trypanosoma*-positive organs/total number of organs. Between brackets: 95% confidence interval (95% CI).

2.5 Discussion

This study is consistent with previous findings that trypanosomes in Australian marsupials comprise a heterogeneous community, with low levels of host specificity and no evidence of restricted geographical distribution. *T. copemani* has been found in the blood of quokkas and Gilbert's potoroos from Western Australia (Austen *et al*. 2009), in koalas from Queensland (McInnes *et al*. 2011b), and in wombats from Victoria (Noyes *et al*. 1999). *T. gilletti* and numerous closely related trypanosome genotypes have been reported in the blood of koalas from Queensland (McInnes *et al*. 2011a, McInnes *et al*. 2011b) and woylies from Western Australia (Averis *et al*. 2009, Paparini *et al*. 2011). *T. sp* H25, which was found to cluster on different phylogenies with *T. cruzi*, has been reported in the blood of an eastern grey kangaroo from Victoria (Noyes *et al*. 1999) and in possums from Western Australia (Paparini *et al*. 2011). This study has found two new *Trypanosoma* genotypes (G1 and G2: Clade A or *T. copemani* clade) in woylies, quendas, chuditch and common brush tailed possums; five new genotypes (G3-G7: Clade B or *T. vegrandis* clade) in woylies, western grey kangaroos, quendas, tammar wallaby and chuditch; and G8 (Clade C or *T.* sp H25 clade) in woylies, banded hare wallabies and boodies from Western Australia. Marsupials belonging to different families in the same locality could be infected with the same trypanosome genotype, as was the case with woylies and tammar wallabies from Karakamia infected with trypanosomes from Clade B (data not shown). It has been shown that host switching facilitated by ecological fitting, is frequently used for trypanosomes jumping between hosts that share ecological niches (Ferreira *et al*. 2008, Maia da Silva *et al*. 2010). The fact that Australian trypanosomes are able to infect different species of marsupials (lack of host specificity) may represent multiple host switching events through their evolution and this may complicate the understanding of the ecological

interactions between trypanosomes and their marsupial hosts.

To infer phylogenetic relationships between the eight trypanosome genotypes found in this study and previously described species, we sequenced both 18S rDNA and *gGAPDH* genes. The use of partial 18S rDNA sequences alone is considered inadequate for inferring deep levels of phylogenetic relationships and additional gene sequences are needed to help resolve polytomies in the Trypanosomatidae (Hamilton *et al*. 2007, Hamilton *et al*. 2011, Lymbery *et al*. 2011). Phylogenetic trees inferred in this study using both loci (18S rDNA and *gGAPDH*) yielded almost identical topologies, and the general branching pattern was in agreement with those shown in previous analyses based on single and combined 18S rDNA and *gGAPDH* sequences (McInnes *et al*. 2009, Paparini *et al*. 2011, Hamilton *et al*. 2012). This study showed two of the genotypes (G1 and G2) found in woylies, quokkas, chuditch, quendas and common brush tailed possums, firmly clustered in a monophyletic assemblage with different genotypes of *T. copemani* previously described in quokkas, Gilbert's potoroos and koalas suggesting that both G1 and G2 might belong to this *Trypanosoma* species. The genetic distances between G1 and *T. copemani* were lower than the genetic distances between G2 and *T. copemani* suggesting a closer genetic relationship between G1 and all previously described genotypes within the *T. copemani* clade (Clade A). Although the genetic distances between G1 and G2 (0.012) are not large enough to suggest that they are two different species (the genetic distances between two closely related species *T. cruzi* and *T. marinkellei* is 0.023), it was surprising that both genotypes exhibited significant differences in their biological behaviour in the host - only G2 was able to infect host tissues. Unfortunately, previous studies describing *T. copemani* in Australian marsupials have not investigated the presence of trypanosomes in host tissues (Paparini

et al. 2011, Austen *et al*. 2009).

The five remaining genotypes (G3-G7) clustered together in a new clade (Clade B), described as "*T. vegrandis* clade" in Chapter 4 (Thompson *et al*. 2013). The closest relative to trypanosomes within this clade was *T. gilletti,* although it always clustered in a separate clade and the genetic distances between this these species and all trypanosomes from *T. vegrandis* clade (Clade B) were greater than distances between the closely related species *T. cruzi* and *T. marinkellei*. Analysis of a shorter region of the 18S rDNA sequences, including previously described trypanosome sequences from a chuditch and woylies in Western Australia (Smith *et al*. 2008), placed these genotypes within the *T. vegrandis* clade suggesting a high heterogeneity within this clade. This heterogeneity might be related to different traits in the evolution of these trypanosomes along with the different marsupial host species that they infect. Although, *T. copemani* and *T. vegrandis* clade trypanosomes shared a common ancestor with *T. pestanai*, a parasite reported previously from badgers from Europe (Lizundia *et al*. 2011), the genetic distances between them were large.

G8 (Clade C) was almost identical to *T. sp* H25 previously described in an eastern grey kangaroo, with a genetic divergence of only 0.003. Very high similarities in the 18S rDNA and g*GAPDH* sequences between both isolates confirmed that they indeed were the same species. Both *T. sp* H25 and G8 clustered within a clade containing *T. cruzi*, *T. marinkellei* and *T. rangeli* - all trypanosomes from South America (Stevens *et al*. 1999). The placement of this Australian trypanosome at the periphery of the "*T. cruzi* clade" may reflect an ancient shared evolutionary history between these trypanosomes. Two different hypotheses have been raised to explain the origin and evolution of

trypanosomes from the *T. cruzi* clade. The first one is the Southern super-continent or Gondwana hypothesis that suggests trypanosomes belonging to this clade might have originated a long time ago in marsupials on the Southern super-continent or Gondwana comprising present day Antarctica, Australia and South America (Stevens *et al*. 2001). However, more recent findings of bat trypanosomes from the old world clustering within the *T. cruzi* clade failed to support this hypothesis (Hamilton *et al*. 2009). Instead, a new hypothesis was raised, known as the bat seeding hypothesis, which suggests that *T. cruzi* clade trypanosomes were originally bat parasites that evolved from within a larger clade of bat trypanosomes and subsequently made the switch into terrestrial mammal hosts in both the new and old worlds (Hamilton *et al*. 2012). The fact that this study found that all genotypes within *T. copemani* and *T. vegrandis* clades share a common ancestor with *T. pestanai* from Europe indicates that although the mammals of Australia have been geographically isolated, their trypanosomes have not, supporting the "bat seeding" hypothesis. Moreover, previous descriptions of other trypanosomes from Australian wildlife that shared common ancestors with trypanosomes from all over the world including *T. theileri* from Germany, *T. cyclops* from Malaysia, and *T. granulosum* from Portugal (McInnes *et al*. 2011b) questioned the veracity of the Southern super-continent hypothesis.

Using a similar nested PCR from the 18S rDNA locus as used in previous studies, this study found substantially higher levels of trypanosome infection: 67% prevalence in blood from nine different species of marsupials. In contrast, Paparini *et al*. (2011) reported a prevalence of only 5% in blood from 11 species of Western Australian marsupials. These differences may be partly attributed to the sensitivity of the PCR used and to the temporal fluctuation of trypanosomes in blood during the natural course of

infection. Most of the trapped and released animals screened in the present study may have been in the active acute phase of infection where animals present high parasitemias easily detectable by PCR or microscopy. Previous studies have shown that in the early acute stage of *T. cruzi* infection, diagnosis is straightforward due to high levels of parasitemia but in the chronic stages, low parasitemia often precludes detection in fresh blood (Russomando *et al*. 1992, Vago *et al*. 2000, Lane *et al*. 2003, Campos *et al*. 2010). Another possible explanation for differences in prevalence may be due to the capacity of Australian trypanosomes to migrate to different organs in the host; in some stages of infection, parasites may be more frequently found in tissues than in peripheral blood, reducing the chances of detection by PCR. This could explain why in three woylie carcasses from which we could collect both blood and tissues, trypanosomes were detected by PCR in tissues, but not in blood. This study also found a relatively high prevalence of mixed infections in woylies, but not in other host species. However, due to the low number of samples examined from the other marsupial species, except blood samples from western grey kangaroos and chuditch, the possibility that mixed infections can also occur in other species cannot be completely excluded.

The fact that genotype, clade or species-specific molecular tools were not used previously (Smith *et al*. 2008, Paparini *et al*. 2011) may have masked the presence of less abundant trypanosomes in mixed infections. Results of PCR and sequencing using the 18S rDNA generic primers compared with clade-specific primers showed that more abundant *T. copemani* genotypes (Clade A) in mixed infections masked the presence of less abundant *T. vegrandis* genotypes (Clade B) and *T. sp* H25 genotypes (Clade C). These results confirm the importance of the use of specific primers for rapid identification of trypanosomes in naturally infected marsupials, especially since

sequencing directly from amplicons alone may not be able to detect mixed infections.

Although all trypanosome genotypes were found in the blood, only some genotypes were seen to colonise tissues. This suggests a genetic basis for the heterogeneity in the biological behaviour of trypanosomes in their marsupial host. We also found, for the first time in trypanosomes infecting Australian marsupials, a differential tissue tropism of trypanosomes within the *T. copemani* and *T. vegrandis* clades. Several studies have demonstrated that the genetic variability of *T. cruzi* may determine the different histotropism observed during infections and consequently the diverse clinical forms of the disease (Andrade *et al*. 1999, Vago *et al*. 2000, Macedo *et al*. 2004, da Silva Manoel-Caetano *et al*. 2008, Ramírez *et al*. 2010). However, the influence of the host genetic background in this process has also been demonstrated (Andrade *et al*. 2002, Freitas *et al*. 2009). Two previous studies found a differential tissue tropism of two genetically different clones of *T. cruzi* in experimentally infected mice (Botero *et al*. 2007) and the presence of a mixed infection in a chagasic patient from Colombia, consisting of two genetically diverse *T. cruzi* populations, one of them infecting the oesophagus and the other infecting the heart (Mantilla *et al*. 2010).

It has been demonstrated that trypanosome multiplication in the vertebrate host occurs extracellularly in the bloodstream or intracellularly in tissues. *T. lewisi* replication occurs extracellularly in visceral capillaries, while *T. cruzi* uses host cells to replicate intracellularly and escape from the immune system to continue its life cycle, resulting in the infection of many tissues and organs (De Souza *et al*. 2010). Our finding of DNA of some genotypes representative of all three trypanosome clades in different marsupial organs, in addition to the discovery of intracellular amastigotes in some tissues where

T. copemani G2 DNA was present (see Chapter 3), confirms that at least this genotype is able to migrate to different tissues in the host, and once there, is capable of invading cells. It has been shown that intact amastigotes are not easy to find during a chronic *T. cruzi* infection, possibly due to the damage to host tissues and destruction of intracellular parasites caused by a strong inflammatory process (Higuchi *et al*. 1993, Zhang *et al*. 1999). This could explain why the presence of amastigotes in marsupial tissues was not a common finding. However, there is a need to isolate trypanosomes in culture and to infect cells *in vitro* in order to confirm the development of Australian trypanosomes inside host cells.

In summary, this study highlights the wide genetic diversity and lack of host specificity of trypanosomes within Australian wildlife and reveals for the first time the capacity of Australian trypanosomes to migrate to different organs in the host.

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Chapter 3

Trypanosoma copemani infection and polyparasitism associated with the population decline of the critically endangered Australian marsupial, the brush tailed bettong or woylie (Bettongia penicillata)

3.1 Abstract

The Brush-tailed Bettong or woylie is on the brink of extinction. This marsupial species has undergone a dramatic overall 90% reduction in abundance despite no apparent increase in the number or type of predators in the region and no apparent decrease in natural resources. The reasons for the decline are currently unknown. However, disease, possibly caused by trypanosome parasites, which are known to be at high prevalence levels, have been under consideration as an important factor associated with the decline. The diversity and prevalence of infections with *Trypanosoma* parasites was investigated and compared between woylies at stable and declining populations. Pathological signs associated with *Trypanosoma* infections were also investigated. Results showed high rates of infection with *T. copemani* (96%) in woylies from the declining population at the Upper warren region. However, in the stable population at Karakamia Sanctuary, *T. vegrandis* was predominant (89%). Mixed infections were common in woylies from the declining but not from the stable population and histopathological findings associated with either mixed or single infections involving *T. copemani* G2 showed pathological changes similar to those seen in *Didelphis marsupialis* infected with the pathogenic *T. cruzi* in South America: myocarditis and muscle degeneration. These results provide evidence for the potential role of trypanosomes in the decline of a formerly abundant marsupial that is now critically endangered, and contribute with valuable information towards directing management decisions for endangered species where these parasites are known to be present at high prevalence levels.

3.2 Background

Australia accounts for one third of the world's mammal species, which have become extinct, the majority being marsupials (McKenzie *et al*. 2007). The woylie, a small potoroid marsupial occupied most of the southern half of the Australian mainland before European settlement in 1788. However, by the 1970s, woylies were listed as critically endangered due to a drastic reduction in abundance from habitat destruction and introduced predators (Orell. 2004, Van Dyck *et al*. 2008). The geographical distribution of the woylie became confined to three locations in Western Australia (WA); the Upper Warren Region, Tutanning Nature Reserve and Dryandra Woodland (Figure 1) (Van Dyck, and Strahan. 2008). Significant conservation efforts, which included predator control and captive breeding and release, led to an increase in abundance from the mid 1970s into the early 2000s. As a consequence of these efforts, woylies were the first Australian taxon to be removed from the endangered species list in 1996 (Start *et al*. 1998). However, since 1999, the three remaining populations have undergone a dramatic overall 90% reduction in abundance, despite no apparent increase in the number or type of predators in the region and no obvious decrease in natural resources. In particular, in the Upper Warren region, the declines have ended up in a net loss of 95% (Wayne *et al*. 2013b). As a consequence of these population declines, woylies have been listed once again as critically endangered by the International Union for Conservation of Nature (IUCN) (Wayne. 2008, Groom. 2010). In efforts to preserve the species, seven translocated populations were established within its previous range (Start *et al*. 1998). However, Karakamia wildlife sanctuary, a predator-proof fenced reserve located 50 km north-east of Perth, is the only substantial translocated population of woylies that has remained stable in Western Australia (Figure 1).

The cause of the second wave of the decline of the woylie is unknown. Predators have been suggested again to play an important role in the decline (Wayne *et al*. 2013b). However, the areas and frequency of ground-based fox-baiting were increased throughout the 1980s and 1990s (Wayne *et al*. 2006), and a broad-scale aerial baiting began in 1996 and is ongoing as part of the 'Western Shield' conservation program (Friend *et al*. 2004). Unfortunately, any substantial recovery of the woylie has not been sustained to date, and one of the three remaining indigenous populations now appears to be extinct (Wayne *et al*. 2013b). Human activity and habitat lost have also been suggested to be influencing the decline, although preliminary investigations have shown it is unlikely (Wayne. 2008). Disease, possibly caused by *Trypanosoma* parasites shown to be at high prevalence levels (Thompson *et al*. 2014), has been under consideration as an important factor associated with the decline (Smith *et al*. 2008). Trypanosomes range from pathogenic species to those that are considered harmless to the vertebrate host. However, non-pathogenic trypanosomes may be potentially pathogenic in the presence of stress, poor nutritional status, and concurrent infections (Hussain *et al*. 1985, Doherty *et al*. 1993, Seifi. 1995). Although several species and genotypes of *Trypanosoma* have been found infecting Australian wildlife, only one species has been implicated with the decline and extinction of an indigenous Australian mammal. This was the case of the exotic *T. lewisi,* which was introduced into Christmas island with fleas on ship rats (*Rattus rattus*) and was suggested to be a likely cause of the extinction of the native rat, *R. macleari* using ancient DNA sequencing of museum specimens of both native and ship rats collected from the island during this time (Pickering et al. 1996, Wyatt *et al*. 2008, MacPhee *et al*. 2013). *T. lewisi*-like trypanosomes have also been described from the Western Australian endangered marsupial, the dibbler (*Parantechinus apicalis*) (Averis *et al*. 2009). However, there is a complete lack of information about the

potential pathogenicity of this trypanosome and its influence on the population decline of the dibbler.

Considering the pathogenic potential of trypanosomes when encountering new host species or during concomitant infections, it is becoming increasingly important to determine the diversity of trypanosomes infecting the woylie. Therefore, the aim of this study is to investigate and compare the diversity and potential pathogenicity of trypanosomes infecting the woylie in declining and stable populations in the Upper Warren region and Karakamia Sanctuary respectively.

3.3 Materials and methods

3.3.1 Woylie samples

A total of 494 blood samples and 154 tissue samples previously collected from woylies (Chapter 2) were used. 237 blood samples were from woylies in the stable population in Karakamia Sanctuary and 257 from woylies in the declining population in the Upper Warren Region. Tissues were collected from twenty-seven fresh or frozen carcasses of woylies and at least two of the following tissues were collected from each carcass: spleen, liver, lung, heart, kidney, brain, oesophagus, tongue, skeletal muscle and bone marrow. All tissue samples were extensively washed with phosphate buffered saline (PBS) and stored in 10% formalin for histopathological analysis.

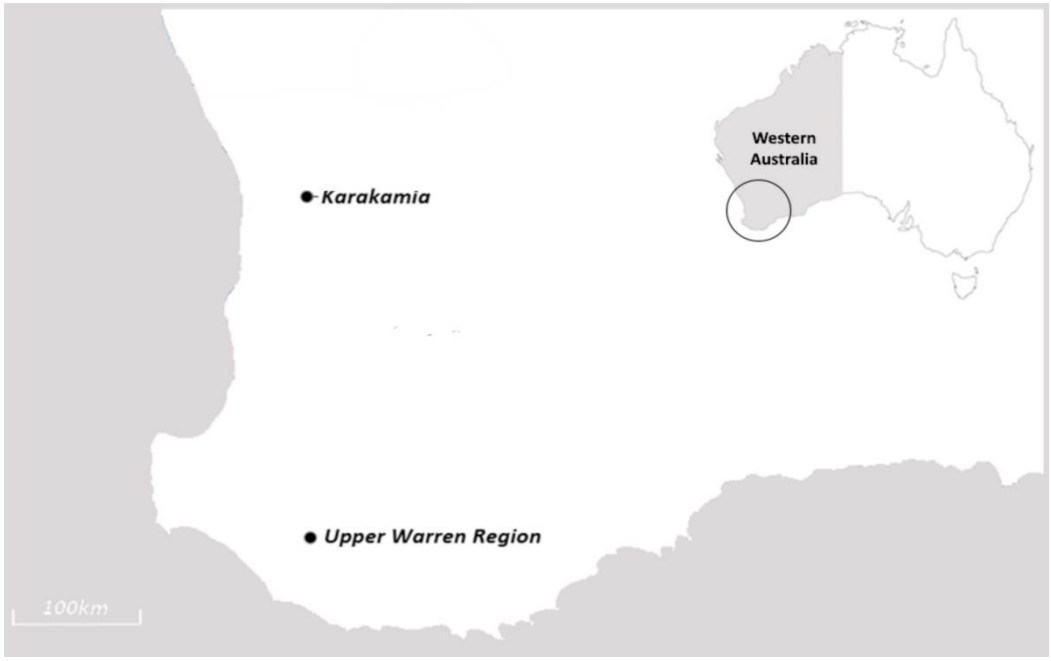

Figure 1. Geographical origin of the woylies analysed in this study

3.3.2 Detection of trypanosomes in blood and tissues by PCR

Trypanosoma clade-specific primers and PCRs used in Chapter 2, which amplify *T. copemani* (Clade A), *T. vegrandis* (Clade B) and *T.* sp H25 (Clade C) genotypes per separate, were used to determine the species of *Trypanosoma* present in each sample. PCR reactions for all blood and tissue samples were performed as described in Chapter 2. Prevalence of infection with trypanosomes was expressed as the percentage of samples found positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa *et al.* 2000). When samples were infected with *T. copemani* (Clade A) genotypes, further sequencing of the PCR products was performed to differentiate between *T. copemani* G1 and G2 as described in Chapter 2.

3.3.3 Histopathology

PCR positive tissues showing single infections with genotypes from *T. copemani*, *T. vegrandis* and *T. sp* H25 clades and also tissues infected with more than one *Trypanosoma* species were paraffin embedded and 3 μm-thick sections were cut and stained with hematoxylin and eosin (H&E). Each section was examined microscopically for inflammatory lesions and the presence of trypomastigotes in blood vessels and amastigotes inside tissue cells. Histopathology sections of 10 tissues taken from two non-infected road kill woylies were used as controls.

3.3.4 Stable population vs. declining population

Prevalence of the different genotypes of trypanosomes and pathological findings were compared in woylies from the stable and declining populations.

3.3.5 Statistical Analysis

Statistical significance was evaluated using the Student's t-test (95% confidence interval) with the GraphPad Prism version 6.0 for Mac (GraphPad Software, San Diego California USA, www.graphpad.com). *Trypanosoma* infection data in euthanised and road kill woylies was compared using the Fisher's exact test.

3.4 Results

3.4.1 Comparison of trypanosome infections between the stable and declining population

Woylies showed a rate of infection of 68% in the blood of trapped and released animals

and 67% in carcasses of dead and euthanised animals (Table 1). There was a significantly higher prevalence of trypanosome infection in sick and euthanised woylies compared with those found dead on roads; while only four out of 12 road kills were infected with trypanosomes, almost all (nine out of 10) euthanised animals were positive for trypanosome infection (Fisher exact test, P=0.01).

Table 1. Overall prevalence of *Trypanosoma* infection in blood and tissues of woylies

Marsupial species	**Blood samples (%, 95% CI)**	**Carcasses (%, 95%CI)**	**Tissue samples (%, 95% CI)**
Woylie *(Bettongia penicillata)*	335/494 (68, 63-71)	18/27 (67, 46-81)	67/154 (43, 35-51)

A significant difference in the prevalence of infection with genotypes from *T. copemani* (Clade A) and *T. vegrandis* (Clade B) in the stable and declining populations was found (Fisher exact test, P=0.0001). Among positive samples, the genotypes most frequently found in the stable population were from *T. vegrandis* (Clade B), present in 89% (95% CI=82-94) of the samples (including mixed infections involving this species). In contrast, genotypes from *T. copemani* (Clade A) were most frequently found in the declining population, present in 96% (95% CI=93-98) of the samples (including mixed infections involving this species). *T.* sp H25 G8 (Cade C) presented the lowest prevalence of infection in both declining and stable populations and mixed infections were found more frequently in the declining than in the stable population (Fisher exact test, P=0.001) (Figure 1, Table 2).

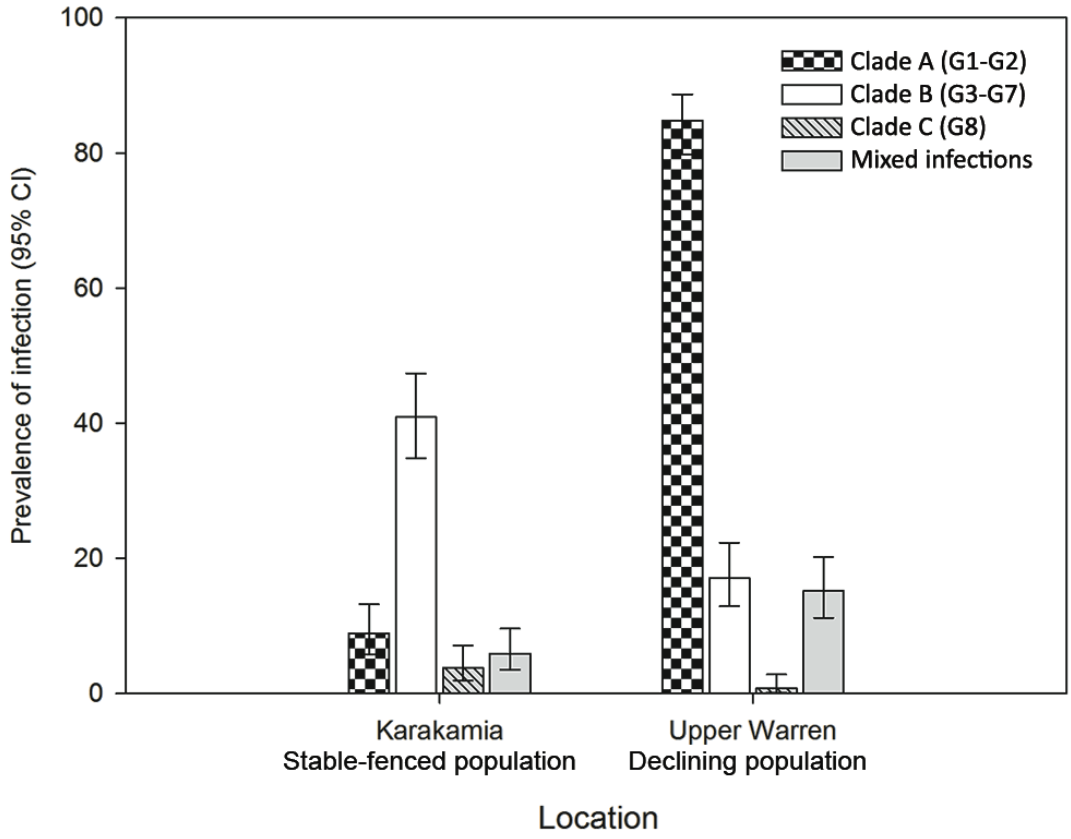

Figure 1. Prevalence of infection with trypanosomes within the different clades in woylies from the stable and declining populations. Clade A (*T. copemani*), Clade B (*T. vegrandis*), Clade C (*T.* sp H25). 95% confidence intervals (95% CI)

Table 1. Overall prevalence of *Trypanosoma* infection in blood and tissues of woylies

Woylie *(Bettongia penicillata)*	***T. veg***	***T.* sp H25**	***T. cop***	***T. cop* + *T. veg***	***T. veg* + *T.* sp H25**	***T. cop* + *T. veg* + *T.* sp H25**
Stable Population	83/109 (76%)	2/109 (2%)	9/109 (9%)	7/109 (6%)	2/109 (2%)	5/109 (5%)
Declining Population	5/226 (2%)	0/226 (0%)	180/226 (80%)	37/226 (16%)	1/226 (1%)	1/226 (1%)

* *T. veg*: *T. vegrandis* genotypes (Clade B); *T. cop: T. copemani genotypes* (Clade A); *T.* sp H25: *T.* sp H25 G8 (Clade C).

3.4.2 Histopathology

All tissue samples examined for histopathology were obtained from euthanised woylies and were collected immediately after euthanasia. Tissues from road kills and from woylies that died naturally with no apparent signs of injury or trauma and with minimal post-mortem decomposition were also used. Of the 13 woylie carcasses that were shown to be infected with trypanosomes by PCR, all had poor body and coat conditions and heavy infestation with ectoparasites (lice and ticks). Eight carcasses that were found polyparasitised with genotypes from all clades exhibited areas of mild to severe alopecia accompanied by multifocal areas of keratinocyte hyper eosinophilia with nuclear condensation (epithelial necrosis), and loss of structure and focal basophilic debris in the underlying dermis (dermal necrosis) (Figure 2). Six of these carcasses were from the declining population in the Upper warren region and two of them from the stable population at Karakamia Sanctuary.

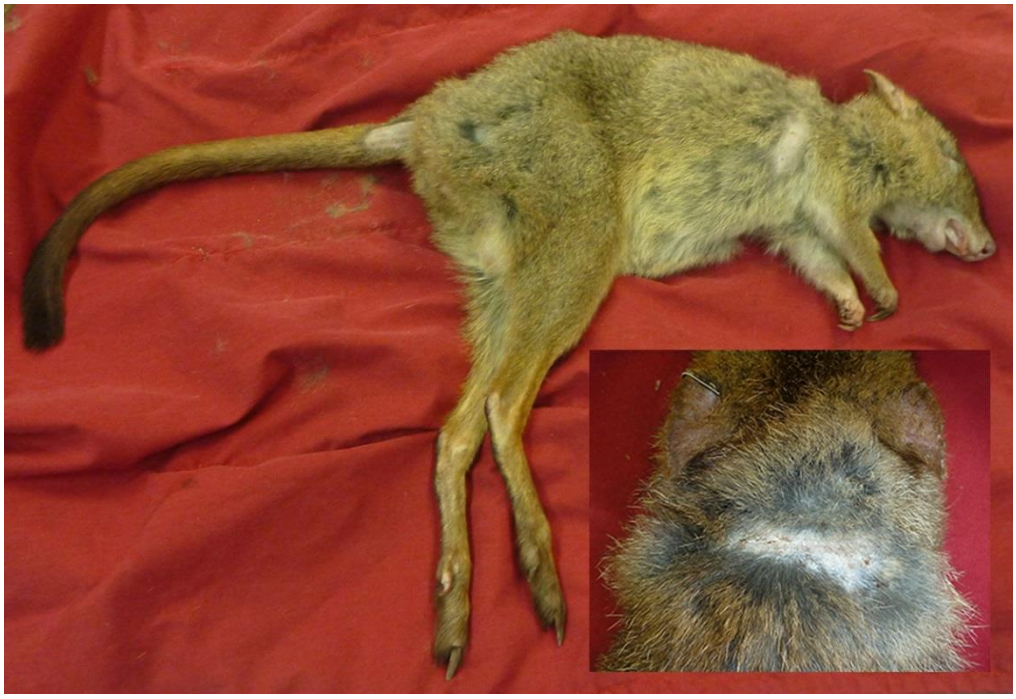

Figure 2. Multifocal alopecia in a woylie from the Upper warren region

Tissue sections infected with genotypes either from *T. vegrandis* or *T.* sp H25 clades, showed an absence of tissue lesions associated with infection. However, a mild inflammatory process was seen in some tissues with a dual infection of genotypes from these two trypanosome species.

A moderate to marked inflammatory process was seen in tissues infected either with *T. copemani* G2 or in co-infections involving this genotype. This process consisted, in general, predominantly of plasma cells, lymphocytes, macrophages, neutrophils and mast cells. The pathology seen in heart sections of three woylies showed a multifocal, severe and chronic pyogranulomatous myocarditis, epicarditis and endocarditis accompanied by muscle degeneration and necrosis (Figures 3A, B and C) similar to that seen in *T. cruzi* infections. One heart sample presented mild right atrial enlargement and occasionally multifocal mild mineralisation.

Tongue and oesophagus were tissues commonly infected. Multifocal, chronic, pyogranulomatous oesophagitis and glossitis, both accompanied by prominent skeletal muscle degeneration was also seen in these tissues (Figures 3D and E). Inflammatory cells were occasionally observed clustering around blood vessels (Figure 3F). Trypanosomes were not observed intravascularly, but structures suggestive of amastigotes were seen in heart tissue of three woylies (Figure 4). Histopathology of 10 sections taken from different tissues of two non-infected road kill woylies showed an absence of tissue lesions.

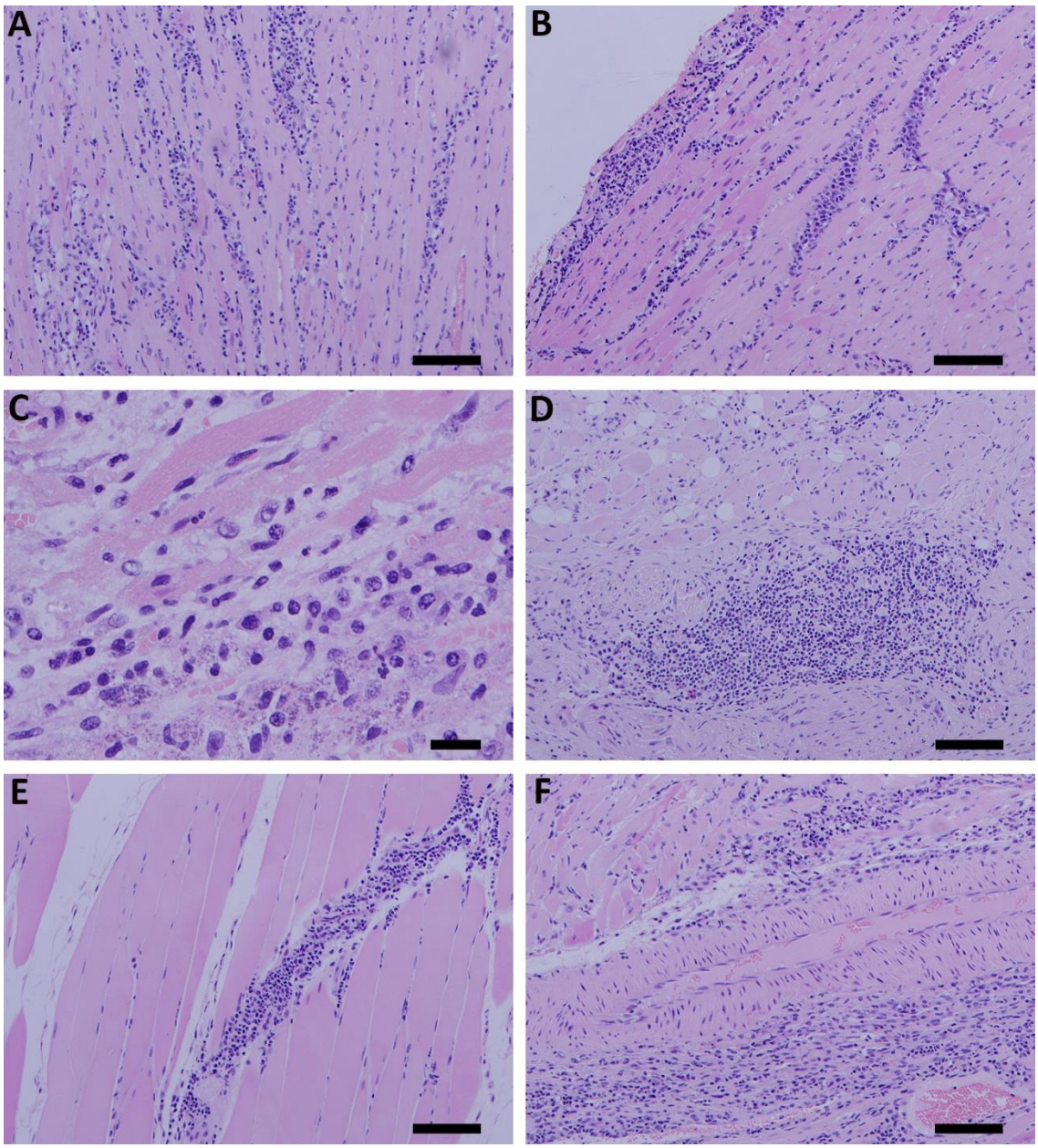

Figure 3. Histopathology of two woylies naturally infected with *T. copemani* G2 (H&E stained). A: Multifocal, moderate to severe, chronic, pyogranulomatous myocarditis and, B: endocarditis; C: mineralisation of heart tissue; D: tongue showing multifocal, moderate, chronic, pyogranulomatous glossitis; E: skeletal muscle degeneration; F: inflammatory cells around a blood vessel. Scale bars: 20 μm.

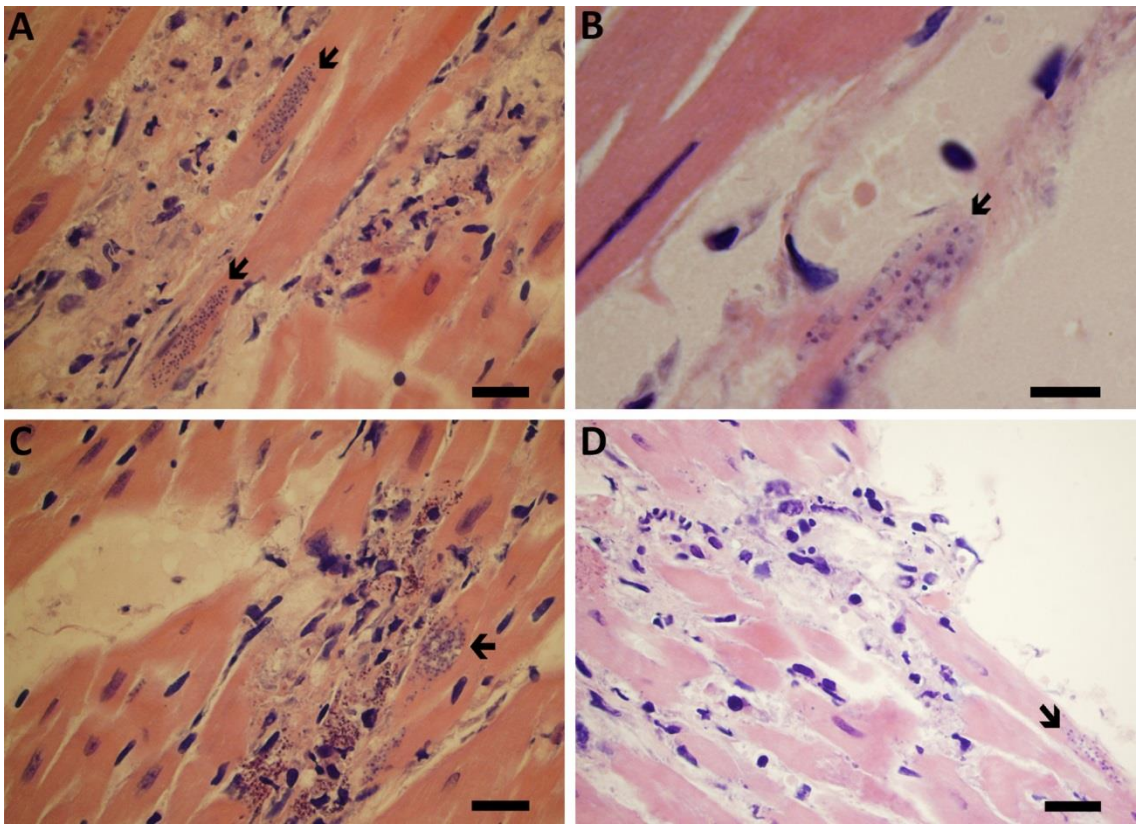

Figure 4. Structures suggestive of amastigotes (arrows) of *T. copemani* G2 in heart tissue positive by PCR (H&E stained). Arrows: *T. copemani* G2 amastigote clusters. Scale bars A, C, D: 20 μm, B: 10 μm.

3.5 Discussion

Several species of trypanosomes have been described infecting humans, livestock and wildlife. They range from non-pathogenic species to those that are highly pathogenic and are the causative agents of many diseases of medical and veterinary importance, including Chagas disease in South America and sleeping sickness and Nagana in Africa (Hamilton *et al*. 2007). *Trypanosoma cruzi*, the agent of Chagas disease in humans has also been described naturally infecting several marsupial species in South America (Rozas *et al*. 2007). Despite a few studies that have shown pathological signs in

marsupial tissues, a lack of information regarding the pathogenicity of this parasite in wild animals is evident (Legey *et al*. 2003). Similarly, some trypanosomes, normally considered as non-pathogenic, are capable of inducing detrimental effects in the host, particularly when the parasite encounters new or naïve host species following their introduction into a new habitat (Maraghi *et al*. 1989) or when an infected host is exposed to additional or increased levels of stress (Brown *et al*. 2000, Wyatt *et al*. 2008). This was the case with the introduction of *Trypanosoma lewisi* to immunologically naïve rodent hosts on Christmas Island, which caused a collapse in the population of the endemic rat *Rattus macleari* to the point of complete extinction (Pickering *et al*. 1996, Wyatt *et al*. 2008). Studies have also shown that *T. theileri*, a non-pathogenic trypanosome of bovids that infects most cattle worldwide, may be considered potentially pathogenic in the presence of stress, gestation, poor nutritional status, and concurrent infections (Hussain *et al*. 1985, Doherty *et al*. 1993, Seifi. 1995). The pathogenicity of Australian trypanosomes has never been explored. However, this study has shown for the first time a correlation between trypanosome infections and pathogenicity in the critically endangered Australian marsupial, the woylie or Brush tailed bettong (*Bettongia penicillata ogilbyi*).

Woylies have undergone an unexpected, rapid and substantial decline (90%) in the last three remaining natural populations throughout Western Australia: Upper Warren region, Tutanning Nature Reserve and Dryandra Woodland, with a total species loss from around 200,000 in 1999 to only 18,000 in 2010 (Wayne *et al*. 2013b). The nature of the decline is unknown, however predation and disease have been considered key factors. Predation by feral cats and foxes were initially associated with most woylie mortalities in the Upper Warren (DEC. 2008). However, a recent study showed the

patterns and magnitudes of the decline could not be solely explained by predation given current evidence from predation activity monitoring in the Upper Warren region and general understanding of cat and fox ecology and movements (Wayne *et al*. 2013b). Interestingly, it was shown that the decline has spread around 4 kilometres per year through the Upper Warren region, leading to the hypothesis that this spatiotemporal pattern of the decline might be due to an agent with limited mobility, such as an infectious diseases moving through the woylie population (Wayne *et al*. 2013b).

Unquestionably, disease has been a key factor in population declines in the past. A Parapoxvirus was implicated in the decline of the red squirrel in the United Kingdom, and the pathogenic fungus *Batrachochytrium dendrobatidis* has been involved in the population decline and extinction of amphibians worldwide including Europe, America and Australia (Berger *et al*. 1998, Skerratt *et al*. 2007, Ramsey *et al*. 2010). Interestingly, in addition to *Trypanosoma*, the woylie has been found infected with different parasites including piroplasms from the genera *Babesia* and *Theileria* (Paparini *et al*. 2012). Despite *Babesia* being pathogenic in livestock and companion animals, the prevalence of infection in the woylie was too low (6.2%) to be considered as a major contributor to the decline. In contrast, the prevalence of infection with *Theileria penicillata* was much higher (80.4%), but microscopic evaluations of woylie blood smears revealed no red cell injury or anaemia suggesting that infections with this parasite might be benign and therefore might not be associated with the decline (Rong *et al*. 2012). Benign infections of *Theileria* have also been observed in another critically endangered marsupial, the Gilbert's potoroo, and in platypuses, where this parasite does not seem to cause any harm to the marsupial host (Collins *et al*. 1986, Lee *et al*. 2009). *Toxoplasma*, an obligate intracellular protozoan parasite, has also been reported

infecting woylies. However, the prevalence of infection was too low (3.61% - 271 woylies examined) to be considered as a major contributor to the decline (Parameswaran *et al*. 2008). *Trypanosoma* infections were reported in woylies for the first time in 2008, and it was suggested that infections with this parasite should be under consideration as an important factor influencing the decline (Smith *et al*. 2008). Smith *et al.* (2008) reported a prevalence of infection of not more than 35% in woylies from Karakamia sanctuary and the Upper Warren region. Interestingly, this study showed higher rates of infection of 68% in woylies from the same localities. This noticeable difference could be explained by an active dispersion of *Trypanosoma* infections in the woylie population, therefore, supporting Wayne *et al*. (2013b) previous hypothesis that suggested that the spreading of the decline might be consistent with an infectious agent disseminating through the woylie population. Unfortunately, the insect vectors of Australian trypanosomes are unknown and therefore the transmission pathway is still to be elucidated. Austen *et al.* (2011) found live trypomastigotes of *T. copemani* in the midgut and faeces of the tick *Ixodes australiensis* collected from Australian marsupials after 3 months of collection. Despite the different developmental changes in morphology that trypanosomes normally undertake in the insect vector gut as part of their life cycle, these were not described for *T. copemani* (Austen *et al*. 2011). Further studies are needed to demonstrate the presence of all developmental stages of *T. copemani* in the tick gastrointestinal tract (such as the non-replicative blood trypomastigote, the replicative epimastigote, and the infective metacyclic trypomastigote) (Tyler *et al*. 2001), as well as to verify that the trypomastigotes in faeces are infective to the vertebrate host.

This study demonstrates, for the first time, a correlation between the presence of Australian trypanosomes and pathological changes in woylie tissues, during single infections with *T. copemani* G2 and also during mixed infections involving two or three trypanosome species. The pattern of inflammatory reactions during these infections was very similar and showed frequent muscle degeneration and occasional necrosis. The pathological changes seen in woylie tissues are similar to those seen in *Didelphis marsupialis* infected with the pathogenic *T. cruzi* from South and North America: myocarditis characterised by inflammatory infiltrates in skeletal muscles and oesophagus (Teixeira *et al*. 2006, Barr *et al*. 1991). It has also been demonstrated that the Australian marsupial, *Trichosurus vulpecula* is susceptible to *T. cruzi* infections under experimental conditions (Backhouse *et al*. 1951). This early study showed *T. cruzi* was able to trigger a strong immune response, similar to the one seen in woylies infected with *T. copemani* G2 that ended in cardiac failure and death of the marsupial host (Backhouse, and Bolliger. 1951). The fact that pathological changes were seen during single and mixed infections involving *T. copemani* G2 may merely reflect the virulence of this genotype. However, pathological changes were also observed in mixed infections involving *T. vegrandis* and *T*. sp H25 (in the absence of *T. copemani*), suggesting alternative scenarios where the woylie immune system may be less efficient at resolving multiple infections or when mixed infections have potentiated pathogenicity. This latter hypothesis regarding heightened pathogenicity during mixed infections is in agreement with a previous study that suggested *T. gilletti* might be potentiating other disease syndromes affecting koalas such as chlamydiosis. McInnes *et al*. (2011b) showed that koalas infected with *T. gilletti* and with signs of chlamydiosis had significantly lower body scores compared to koalas that were not infected with *T. gilletti*. Furthermore, a significantly higher proportion of koalas with mixed

trypanosome infections (*T. gilletti, T. irwini,* and *T. copemani*) did not survive compared to uninfected koalas (McInnes *et al*. 2011b).

Natural mixed infections between different species or genotypes of trypanosomes occur frequently in nature, with both parasite and host factors determining the overall parasitemia, virulence and pathogenicity (Martins *et al*. 2006, Pinto *et al*. 2006, Pollitt *et al*. 2011). Although several studies have reported the intrinsic characteristics of single trypanosome infections, it has not been clearly demonstrated whether the interaction of different trypanosomes in a single host can affect or modify the infection dynamics by either reducing or enhancing parasitemia, virulence and pathogenicity (Reifenberg *et al*. 1997). Rodrigues *et al*. (2010) showed that the combination of two strains of *T. cruzi* was able to trigger both protective inflammatory immunity and regulatory immune mechanisms that attenuate damage caused by inflammation in experimentally infected mice. However, in natural infections, virulence is difficult to predict when interactions are not restricted to a single parasite species, but involve multiple infections as in the case of woylies.

It has been shown that within the Upper Warren region, the woylie decline is most severe and has reached 95% within a 6-year period (Wayne *et al*. 2013b). Our results showed a marked difference in the composition of trypanosome infections between woylies in the stable and the declining population at Karakamia and the Upper warren region respectively. The greater prevalence of *T. copemani* and mixed infections in the declining population of woylies in the Upper warren region, together with the demonstrated potential of these trypanosomes to cause pathological changes in woylies, suggests that infections with trypanosomes within this species could be important

contributors to the dramatic decline of the woylie. Given that we only examined woylies from a single stable population and a single declining population, there is a clear need for further studies to test this hypothesis, although it is important to note that *T. copemani* has been reported in the blood of other critically endangered and vulnerable Australian marsupials including Gilbert's potoroos, and quokkas from Western Australia (Austen *et al*. 2009) and koalas from Queensland (McInnes *et al*. 2011b). A similar pattern of distribution of the different species of *Trypanosoma* in woylies from the stable and declining populations was reported recently. Thompson *et al*. (2014) found a high prevalence of *T. copemani* infections in woylies from the declining population and not from the stable population, and a high prevalence of infection with *T. vegrandis* (100%) in woylies from the stable population at Karakamia sanctuary. Considering the high prevalence of *T. copemani* infections in woylies from the declining population and the inflammatory process observed in tissues, there is an urgent need for future studies to investigate the impact of this parasite on the health of other co-habiting threatened or endangered species such as the quenda (*Isoodon obesulus*), chuditch (*Dasyurus geoffroii*), Dunnart (*Sminthopsis* spp.), Wambenger (*Phascogale tapoatafa*), and Ngwayir (*Pseudocheirus occidentalis*) (Wayne *et al*. 2011).

Interestingly, Wayne *et al.* (2013b) showed a periodicity in the pattern of the woylie declines in the Upper Warren region since 1999. He found the decline occurred for an average of four years (range 3-5 years) with post declining capture rates remaining low for at least four years. Warrup, one of the sampled sites in the Upper Warren region, began the first apparent recovery in 2005, but having recovered to a 40% capture rate, subsequently declined again, beginning in 2009. Following a similar pattern to Warrup,

after four years at low post-decline numbers, two other sampled sites in the Upper Warren region, Camelar and Boyicup, experienced a slight recovery beginning in 2009 (Wayne *et al*. 2013b). The cause of this periodicity in the decline is unknown. However, it might be explained in part by seasonal fluctuation of insect vectors (climate related) and therefore increased parasitemias in woylies when there is abundance of vectors. Alternatively, it can be explained by a chronic condition caused by *Trypanosoma* parasites that might be debilitating the woylie and accounting for a high morbidity and mortality after several years of infection. It has been shown that deaths are common in the latest stage of *Trypanosoma* infections and especially when tissues are compromised. *T. cruzi* for example, presents different stages of infection. An acute stage at the beginning of the infection, characterised by high parasitemias, and followed by a latent stage, which is asymptomatic, and can last for years. Finally, a chronic stage where parasites replicate inside cells in target tissues that usually ends in tissue damage and death. Unfortunately, it is not easy to undertake long-term monitoring of *Trypanosoma* infections in woylies in the wild. However, during this study there was the opportunity to screen monthly eight woylies naturally infected with trypanosomes, for three years. Although, in general infections persisted over the time and parasitemias were always detected by PCR and haemoculture (Thompson *et al*. 2014), woylies infected with *T. copemani* G2 exhibited temporal fluctuations in parasitemia. To explain the disappearance and appearance of parasites in blood, it was suggested that trypanosomes might have migrated to tissues (Thompson *et al*. 2014). Because the average life expectancy of woylies is between 4 to 6 years (Christensen. 1995), there is the need to continue monitoring those woylies to confirm that the woylie trypanosomiasis might progress to a chronic condition that ends up affecting vital organs in the marsupial host.

The reported decrease in the genetic background of woylies cannot be excluded as a possible contributor to the decline. The woylie has suffered genetic bottlenecks due to habitat fragmentation and translocations, and a loss in their genetic variability is evident (Pacioni. 2010). It has been shown that a decrease in the genetic variability at the species level can reduce the fitness of populations and increase their susceptibility to infectious agents. The Tasmanian devil, for example, is a species in which low genetic diversity (Jones *et al*. 2007, Miller *et al*. 2011) has increased susceptibility to disease (Siddle *et al*. 2007). This might be happening also in the woylie, and the reduced genetic variability within the species could explain their greater vulnerability to the pathogenic effects of *Trypanosoma* infections compared with the other marsupials examined.

In summary, the research reported in this chapter has revealed for the first time the pathogenic potential of *T. copemani* either in single or mixed infections and showed a strong association between *Trypanosoma* infections and the dramatic decline of the woylie. These results provide evidence for the potential role of trypanosomes in the decline of a formerly abundant marsupial that is now critically endangered.

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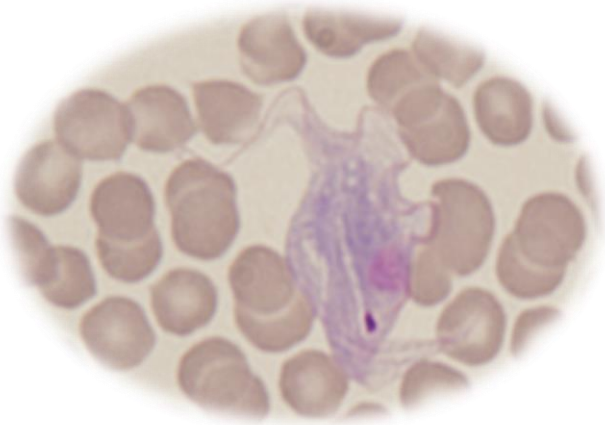
Chapter 4

Isolation of Australian trypanosomes: a comparison of growing requirements and morphology



4.1 Abstract

To better understand the biology and life cycle of trypanosomes infecting Western Australian marsupials, different trypanosome strains were isolated from the blood of woylies, and their growth requirements and morphology were described. Woylie blood was inoculated in culture tubes containing a biphasic medium composed of agar-BHI and horse blood as a solid phase and an overlay of four different media as a liquid phase, RPMI 1640 (Roswell Park Memorial Institute 1640), HMI-9 (Iscove's modified DMEM-based), Schneider's (Schneider. 1964) or Grace's (Grace *et al*. 1966) media. Trypanosomes were observed in 24 haemocultures after eight days post-inoculation when RPMI 1640 was used as a liquid phase medium. PCR results using trypanosome species-specific primers showed that all isolates were *T. copemani* G1 and G2. Most bloodstream trypomastigotes had differentiated into epimastigotes by the first week post-inoculation and spheromastigotes and metacyclic trypomastigotes were observed in two week old cultures. The enhancement of growth by different concentrations of hemin was investigated, and morphological features and social motility behaviour shared with other trypanosomatids are discussed.

4.2 Background

The use of biphasic media containing a solid phase of agar enriched with blood has been shown to be the best method to isolate primary cultures of different and new species of trypanosomes (Stohlman *et al*. 1973, Tanuri *et al*. 1985, Lemos *et al*. 2013). However, establishing and maintaining cultures in the laboratory is laborious and requires finding the optimum medium that provides the best nutritional conditions for growth.

Growth requirements differ according to the species of *Trypanosoma*. HMI-9 medium, based on Iscove's modification (IMDM) of Dulbecco's MEM (Modified Eagles Medium), for example, is the most commonly used to culture blood forms of *T. brucei* and *T. congolense* parasites that replicate in blood of the vertebrate host (Baltz *et al*. 1985, Hirumi *et al*. 1991, Uzureau *et al*. 2013). This medium produced double rates of *T. brucei* replication *in vitro* when compared with RPMI 1640 and Leibovitz**'**s media (Zweygarth *et al*. 1990, Schuster *et al*. 2002). *T. cruzi*, a parasite that replicates in host tissues, is able to grow in different media, but Liver Infusion Tryptose (LIT) is the most widely used (Camargo. 1964, Botero *et al*. 2010, Pereira *et al*. 2011).

Different growth-promoting factors have been used to improve trypanosome isolation and growth rates in culture. A nutritional characteristic of all different species of *Trypanosoma* is that *in vitro* they need a haem-compound as a growth factor. Studies have shown that haemin plays an important role in growth and differentiation of trypanosomes. An increased parasite proliferation in a haemin dose-dependent manner was demonstrated in cultures of *T. cruzi* (Lara *et al*. 2007). Ciccarelli *et al.* (2007) also showed that a concentration of haemin of 5 mg/l yielded an optimum growth of *T. cruzi* cultured under different haemin concentrations.

Furthermore, an uncharacterised trypanosome isolated from an American kestrel (*Falco sparverius*) and *T. theileri* require haemin in the media for *in vitro* growth (Sollod *et al*. 1968, Kirkpatrick *et al*. 1986). The use of growth-promoting factors and different media that mimic the environmental conditions (amino acid composition) in haemolymph of the insect vector, and/or in blood or tissues of the vertebrate host has also been used to obtain different developmental stages of *Trypanosoma* in *in vitro* cultures (Koerich *et al*. 2002).

Trypanosomes are pleomorphic, adopting a variety of forms/stages in the course of their life cycle. The trypomastigote of *T. cruzi* for example, is found in the bloodstream of the vertebrate host and is able to infect cells in tissues where trypomastigotes transform into amastigotes (intracellular stage). Both the trypomastigote and the amastigote can be maintained *in vitro* in axenic media with cells. In contrast, the epimastigote, the form found in the insect vector, is maintained *in vitro* in cell-free axenic medium, and is commonly used in *in vitro* studies because once established in culture it is easy to obtain large numbers of parasites (Nakayasu *et al*. 2009).

Within Australia, several species and genotypes of trypanosomes have been described infecting wildlife. The description of these species has been based on molecular data, host occurrence and morphology of blood forms. However, little is known about trypanosomes life cycle and the different developmental stages present in the invertebrate and vertebrate host. To date, only a few trypanosomes have been isolated in culture from Australian marsupials. *Trypanosoma sp.* H25, *Trypanosoma sp.* H26 and *T. copemani* were isolated from a kangaroo, a wombat and a Gilbert's potoroo respectively and established in culture in Sloppy Evans semi-solid agar culture medium

(Noyes *et al*. 1999, Austen *et al*. 2009). Although both studies described their morphology in culture, little is known about the different developmental stages of these parasites in natural infections and under experimental conditions.

Considering the lack of information regarding Australian trypanosomes, the aims of this study were to (i) isolate Australian trypanosomes in culture; (ii) determine their optimal growth requirements and; (iii) investigate their different developmental forms and morphology in culture, including their ultrastructural organization.

4.3 Materials and Methods

4.3.1 Haemocultures

Considering the high prevalence of infection and the presence of eight different genotypes of trypanosomes in woylies (*Bettongia penicillata*) (Chapter 2), different trypanosome strains were isolated from the blood of this marsupial. Woylies were trapped in small cage traps (20 cm x 20 cm x 56 cm) baited with a mixture of rolled oats and peanut butter. Traps were placed at set intervals (usually 200 m) along tracks in the study sites. Approximately 100µl of blood was collected from the lateral caudal vein from each individual. Cultures were established by inoculation of approximately 100µl of peripheral blood into 25cm^2 tissue culture flasks or in Nunc™ cell culture tubes (Thermo scientific) with biphasic medium containing Brain-Heart Infusion (BHI) medium, agar, gentamicin and 10% defibrinated horse blood as a solid phase, and either RPMI 1640 (Roswell Park Memorial Institute 1640), HMI-9 (Iscove's modified DMEM-based), Schneider's (Schneider. 1964) or Grace's (Grace, and Brozostowski. 1966) media as a liquid phase, each supplemented with 10% of Fetal Cow Serum (FCS)

and Penicillin-Streptomycin (Table 1). Haemocultures were checked every two days under the microscope for motile trypanosomes. When trypanosomes were seen for the first time, cultures were left for one week and then the supernatant was removed and replaced with new liquid medium. The liquid medium was replaced every two weeks in each culture flask. Optimal growth requirements in liquid medium alone were determined after haemocultures were established in biphasic medium. Cultures were maintained in liquid medium by successive passages every three days at 28°C and 5% CO_2.

Table 1. Different media used to grow trypanosomes in culture

Reagent	Concentration
Brain heart Infusion (BHI)	
Agar	11,1gr/L
BHI	22gr/L
Gentamycin	2,4ml/L
Defibrinated horse blood	10%
RPMI 1640	
RPMI 1640 (invitrogen)	10,3g/L
Sodium bicarbonate	2g/L
L-glutamine	0,3g/L
HEPES (15mM)	3,6g/L
Heat inactivated FCS (fetal calf serum)	10%
Penicillin (10000 IU/ml) & Streptomycin (10000 MCG/ml)	10ml/L
HMI-9	
Iscove's modified Dulbecco's MEM (IMDM)	795ml
Hypoxanthine 100 x solution	13,6mg/ml
Bathocuproine Sulphate 100x solution	2,82mg/ml
Cysteine 100x solution	18,2mg/ml
Cysteine 100x solution	18,2mg/ml
Pyruvate 100x solution	11mg/ml
Thymidine 100x solution	3,9mg/ml
ß Mercaptoethanol solution	14µl/L
Heat inactivated HCS (Horse calf serum)	10%
Penicillin (10000 IU/ml) & Streptomycin (10000 MCG/ml)	10ml/L
SCHNEIDER	
Schneider medium (invitrogen)	880ml
Heat inactivated FCS (fetal calf serum)	10%
Penicillin (10000 IU/ml) & Streptomycin (10000 MCG/ml)	10ml/L
GRACES	
Grace's insect medium (invitrogen)	880ml
Heat inactivated FCS (fetal calf serum)	10%
Penicillin (10000 IU/ml) & Streptomycin (10000 MCG/ml)	10ml/L

*Final volume completed with H_2Odd - pH 7.2 and filter sterilised (0.22µn filter)

4.3.2 Trypanosome detection by PCR with species-specific primers

DNA of trypanosomes growing in culture was isolated and the species determined using species-specific PCR's described in Chapter 2. To differentiate between *T. copemani*

G1 and G2, *T. copemani* positive cultures were resubmitted to a second PCR using the generic primers described in Chapter 2 and followed by sequencing. Sequences were aligned with previous known sequences of *T. copemani* G1 and G2 using ClustalX (Thompson *et al*. 1994).

4.3.3 Trypanosome development in culture

Trypanosome growth was monitored under the microscope in all different liquid media (RPMI 1640, HMI-9, Schneider and Grace's) every day for one month. Growth curves of trypanosomes growing in RPMI 1640, Grace's and HMI-9 media were generated. Schneider's medium did not support the growth of both strains, thus growth curves with this medium were not generated. To investigate the growth kinetics in all three media, trypanosomes were counted under the microscope and $1x10^3$, $1x10^4$ and $1x10^6$ parasites were seeded in triplicate wells in a 96 well plate. The number of trypanosomes in each well was calculated using a haemocytometer chamber for eight consecutive days and growth curves were generated using the Prism 6 software.

4.3.4 Evaluation of different concentrations of haemin on growth

Because haem-compounds play an essential role in trypanosome growth, the effect of haemin on growth and differentiation was investigated. Different concentrations of haemin, 2.5mg/L, 5mg/L, 10mg/L, 15mg/L and 20mg/L were added to Grace's and RPMI 1640 media, media that produced the best rate of growth. $1x10^6$ parasites were seeded into 24-well plates containing each medium with differing haemin concentrations. To investigate the growth rate, trypanosomes were counted in a

haemocytometer chamber every day for six consecutive days and mobility and motility were observed.

4.3.5 Light, scanning and transmission electron microscopy

The morphology of trypanosomes from direct thin blood smears and smears of logarithmic and stationary phase cultures were compared. Smears were fixed in methanol and stained with the commercial Diff-Quick staining system for examination by light microscopy.

For scanning electron microscopy (SEM), culture forms were fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS: cell culture medium (pH 7.2), before being mounted on poly-L-lysine coated coverslips, progressively dehydrated through a series of ethanol solutions and critical point dried as previously described (Edwards *et al*. 2011). Coverslips were mounted on stubs with adhesive carbon, coated with 2 nm platinum (Pt) and imaged at 3kV using the in-lens secondary electron detector on a Zeiss 55VP field emission SEM.

For transmission electron microscopy (TEM), trypanosomes were also fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS: cell culture medium (pH 7.2). All subsequent processing was performed in a PELCO Biowave microwave, where samples were post-fixed in 1% OsO_4 in PBS followed by progressive dehydration in ethanol/acetone, before being infiltrated and embedded in the epoxy resin Procure-Araldite. Sections 120nm-thick were cut with a diamond knife and mounted on copper grids. Digital images were collected from unstained sections at 120kV on a JEOL 2100 TEM fitted with a Gatan ORIUS1000 camera.

4.4 Results

4.4.1 Isolation of trypanosomes in biphasic media and identification of Trypanosoma species by PCR

Due to the fact that woylies exhibited high levels of parasitaemia and were detected harbouring all of the different trypanosome genotypes found in this study, additional peripheral blood samples were taken from animals at Karakamia (stable population) and Upper Warren (declining population) and cultured in biphasic medium. Of the 30 haemocultures obtained from the blood of woylies from the stable population, no evidence of protozoa morphologically similar to trypanosomes was detected after 30 days post-inoculation in culture flasks containing either RPMI 1640, Grace's, Schneider's or HMI-9 media as a liquid phase. Furthermore, no trypanosomes were detected in direct peripheral blood smears from these animals, but PCR and sequencing of blood samples inoculated in the biphasic media revealed that 21 of them were infected with trypanosomes from Clade B (*T. vegrandis* clade). Although this study did not use a quantitative PCR, results showed very strong PCR products comparable with PCR products from woylies in the declining population that presented positive smears and haemocultures as shown in Figure 1.

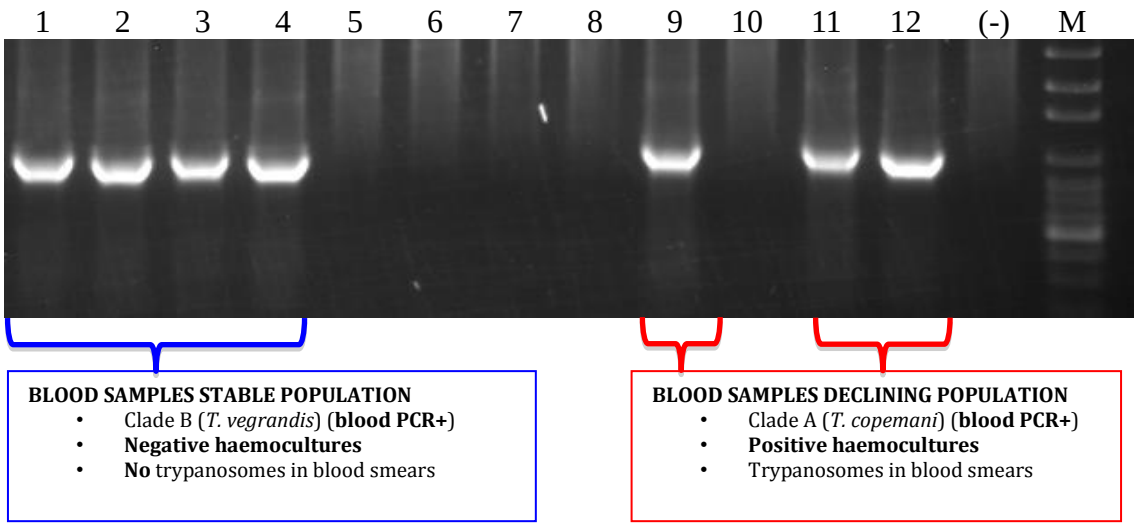


Figure 1. 18S rDNA PCR results from the blood of woylies at the stable and declining populations. M: molecular weight marker (100 bp ladder).

In contrast, of the 30 haemocultures obtained from woylies from the declining population, 24 exhibited trypanosomes in culture flasks containing RPMI 1640 as a liquid phase. Trypanosomes were never detected in haemocultures containing Grace's, Schneider's, and HMI-9 media as a liquid phase. PCR's using species-specific primers revealed the presence of only *T. copemani* G1 and G2 in the haemocultures. The sequence alignment obtained from the *T. copemani* haemocultures showed that 16 haemocultures were *T. copemani* G1 and 8 were *T. copemani* G2 (Figure 2).

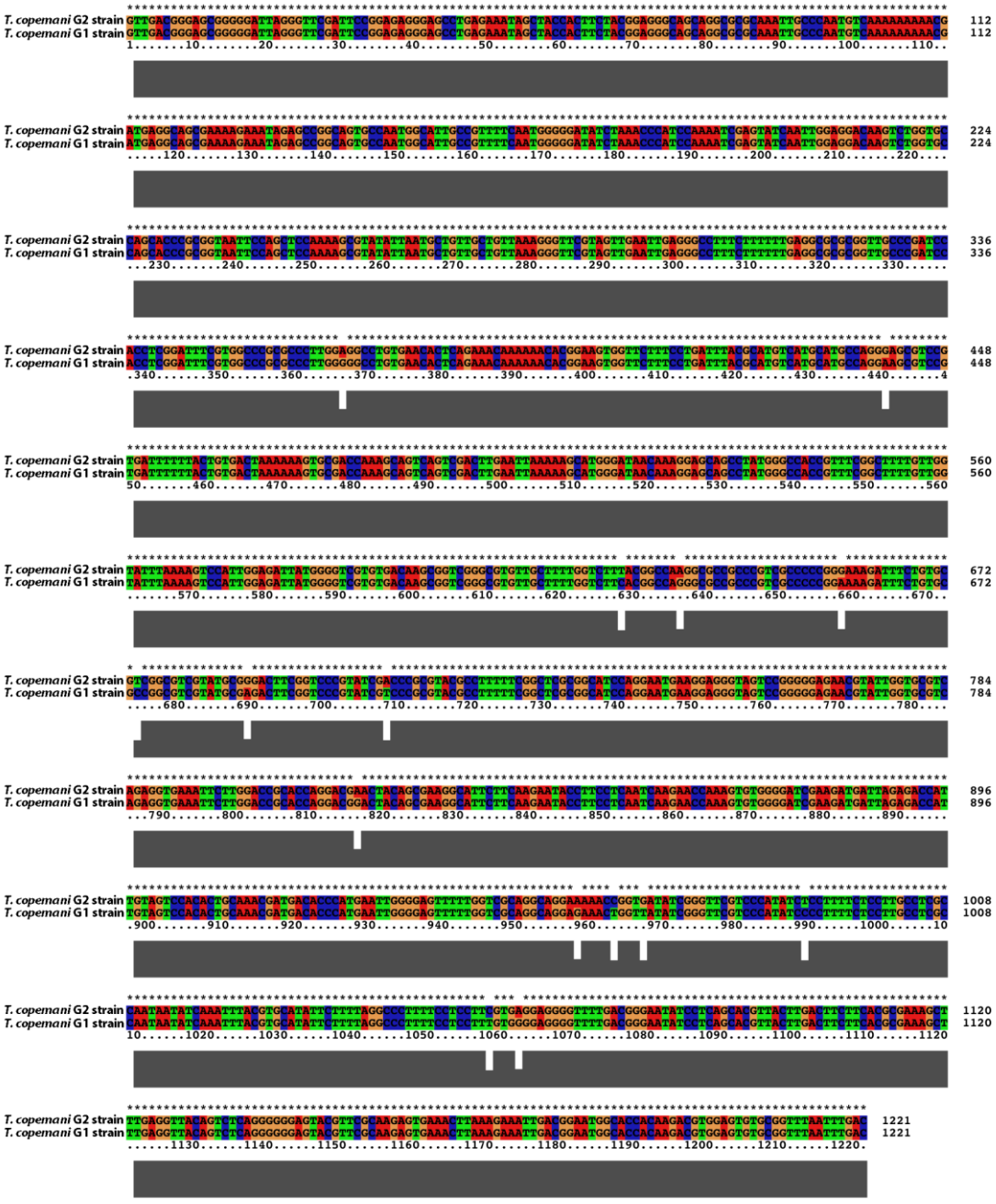


Figure 2. Alignment of the 18S rDNA sequences of *T. copemani* G1 (16 strains), and *T. copemani* G2 (8 strains) isolated from the declining population

Cultures showed emerging trypanosomes after two to three weeks post-inoculation. During these times, trypanosomes started to emerge from the blood-agar and small nests were commonly seen in the liquid phase of the medium. Trypanosomes were also seen attached to the blood-agar surface and actively multiplied there. Nests continuously

grew in size and after two weeks large circular masses of trypanosomes settled down in the surface of the agar and small circular holes were visible in the agar under the microscope.

4.4.2 Trypanosomes growth curves

Both *T. copemani* G1 and G2 were successfully subcultured in biphasic medium for long periods at weekly intervals, but they differed in growth requirements when only liquid media were used. Both *T. copemani* G1 and G2 showed a growth rate significantly higher in RPMI 1640, followed by Grace's and HMI-9 media. However, Grace's and HMI-9 media supported the growth of *T. copemani* G2 better than G1. Trypanosomes growing in RPMI 1640 were seen actively dividing until day 8 post-inoculation. At this time they reached the maximum number and then they started to die (possibly because of the nutrients in the medium were exhausted). Schneider medium failed to support the growth of both strains of *T. copemani*. The doubling growth time of parasites in RPMI 1640 and Grace's media was approximately 2 days. Growth curves of both *Trypanosoma* strains grown separately in the three different media are shown in Figures 3 and 4.

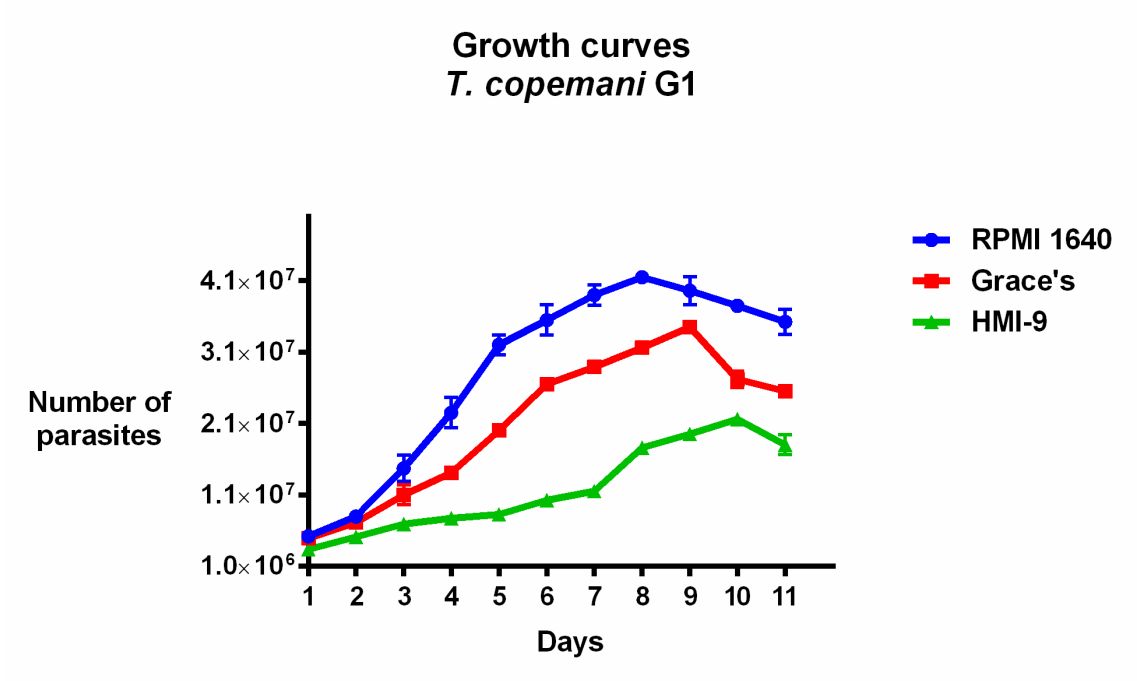


Figure 3. Growth curves *T. copemani* G1 in three different media: RPMI 1640, Grace's and HMI-9. X-axis: number of parasites/ml. Y-axis: days.

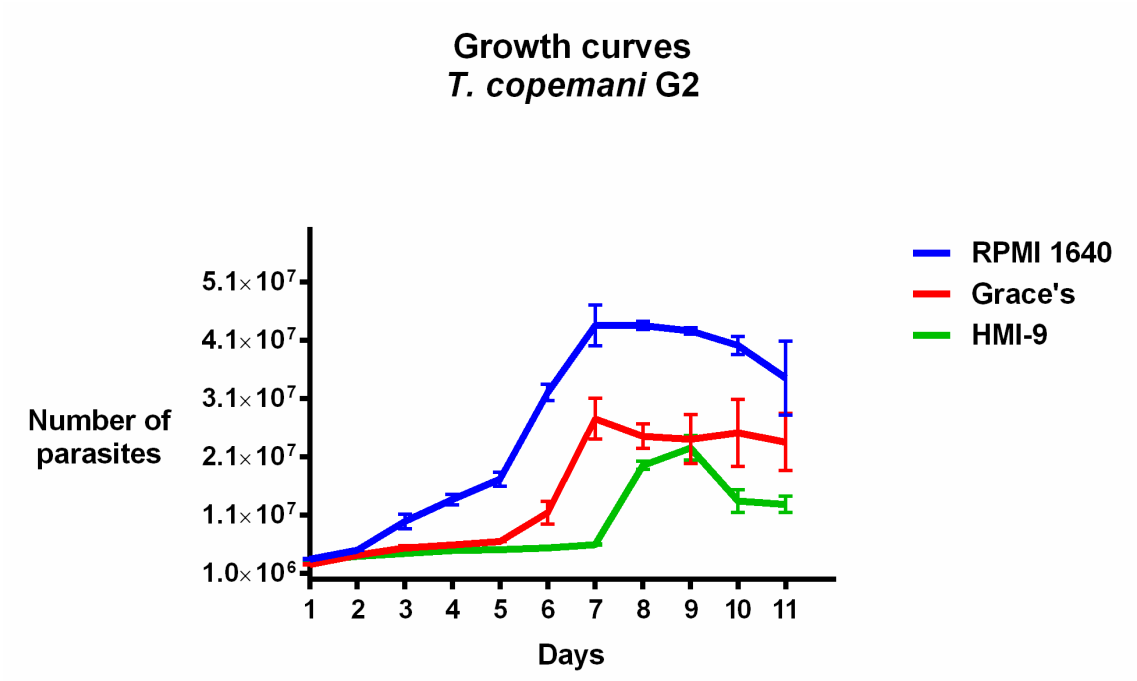


Figure 4. Growth curves *T. copemani* G2 in three different media: RPMI 1640, Grace's and HMI-9. X-axis: number of parasites/ml. Y-axis: days.

4.4.3 Role of haemin in growth

T. copemani G1 and G2 were grown in different concentrations of haemin between 2.5mg/L to 20mg/L. Although, both genotypes of *T. copemani* grew well without haemin, the addition of haemin to the media (at some concentrations) significantly improved growth, mobility and motility. Growth curves could not be generated due to trypanosomes being compacted firmly in nests that could not be homogeneously separated. Therefore, trypanosome growth was followed as a qualitative observation of proliferation (size of nests), mobility (ability to move spontaneously and actively - does not involve displacement) and motility (ability to move spontaneously and actively - involves displacement) under the microscope. The haemin concentrations that produced more and larger nests were 2,5mg/L and 10mg/L for *T. copemani* G1 and G2, respectively (Figure 5). These concentrations improved trypanosome activity in the media in terms of mobility and motility, and free trypanosomes (not in the process of division in nests) moved continuously from side to side in the wells in both *T. copemani* G1 and G2 cultures. However, at the other concentrations of haemin (5mg/L, 15mg/L and 20mg/L) both trypanosomes were not very active and did not divide quickly.

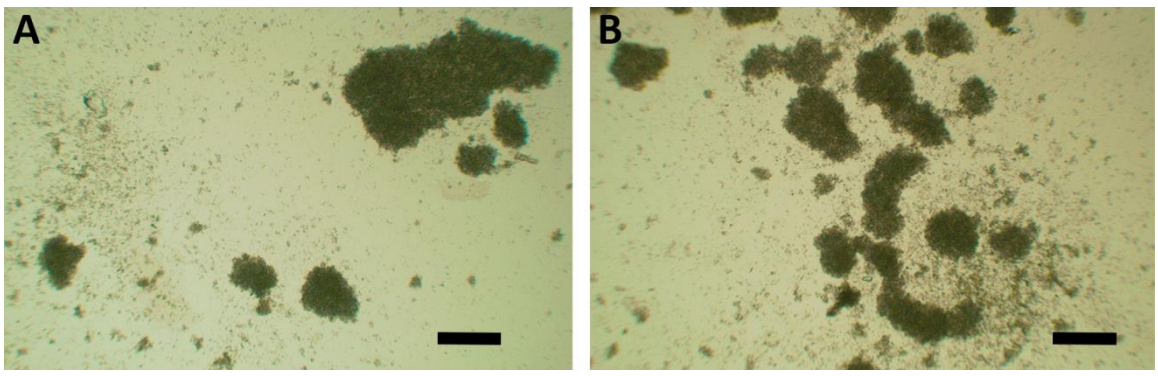


Figure 5. Different size of trypanosomes nests growing in 24 well plates. Scale bars: 30 μm.

Interestingly, trypanosomes formed groups of big and small densely-packed cells (nests) within 24h post-plating. Nests moved across the plate surface, recruiting neighbouring nests (social motility) and forming fusions of large groups as shown in the sequential images (1 to 10) in Figure 6.

Haemin concentrations higher than 10mg/L and 15mg/L decreased the growth rate of *T. copemani* G1 and G2 respectively and also induced morphological changes. Most of the parasites adopted a spherical shape and lost partially or completely the prominent flagella at these haemin concentrations (Figure 7).

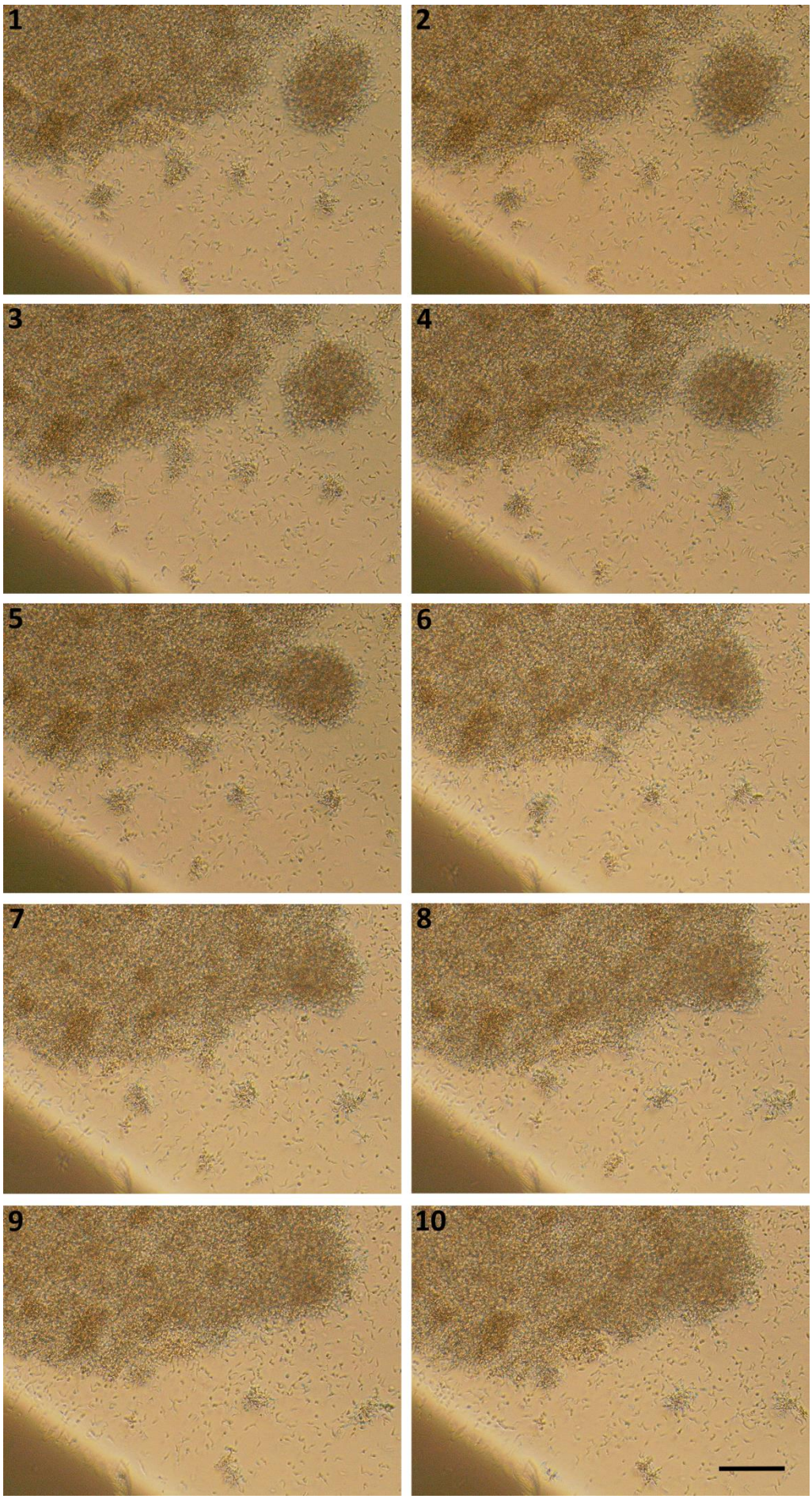

1
2
3
4
5
6
7
8
9
10

Figure 6. Social motility of *T. copemani*. Trypanosome bigger nests recruiting small neighbouring nests. Sequential images (1 to 10) were taken at 2-minutes intervals. Scale bar: 20 μm.

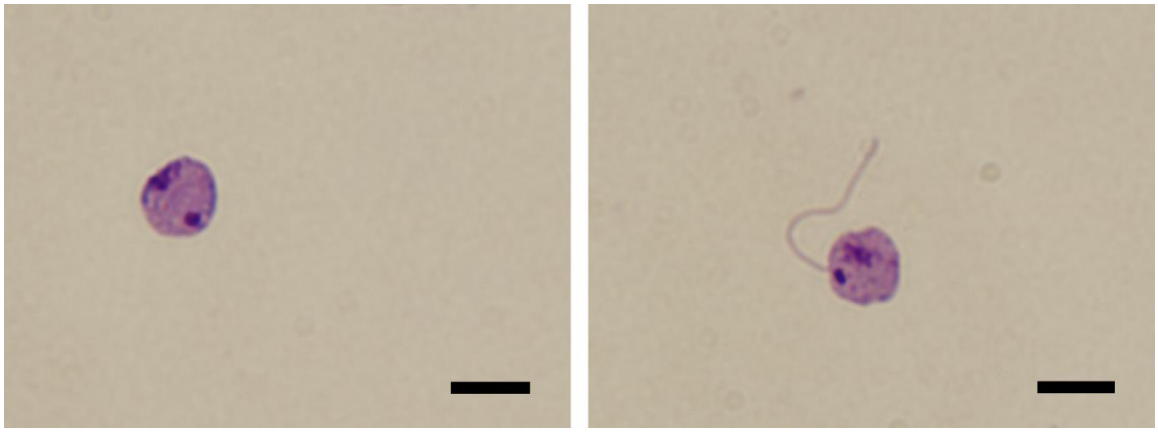


Figure 7. Light microscopy of Diff-quick stained spheromastigotes of *T. copemani* in culture medium containing high concentrations of haemin. Scale bars: 10 μm.

4.3.4 Trypanosomes morphology

Peripheral blood smears from woylies infected with *T. copemani* showed blood trypomastigotes with large body width (6.53±0.31μm over the nucleus) and length (31.82±0.53 μm) (Figure 8A). These forms exhibited a small rounded kinetoplast and an oval and bigger nucleus. The kinetoplast stains densely purple and is positioned close to the nucleus in the posterior end of the cell. The nucleus stains light pink and is located clearly in the center. Trypomastigotes presented also a conspicuous undulating membrane with well-pronounced undulations and flagella that originates just anterior to the kinetoplast. Morphological features were almost identical between both *T.copemani* G1 and G2. Dividing forms were never observed in blood smears.

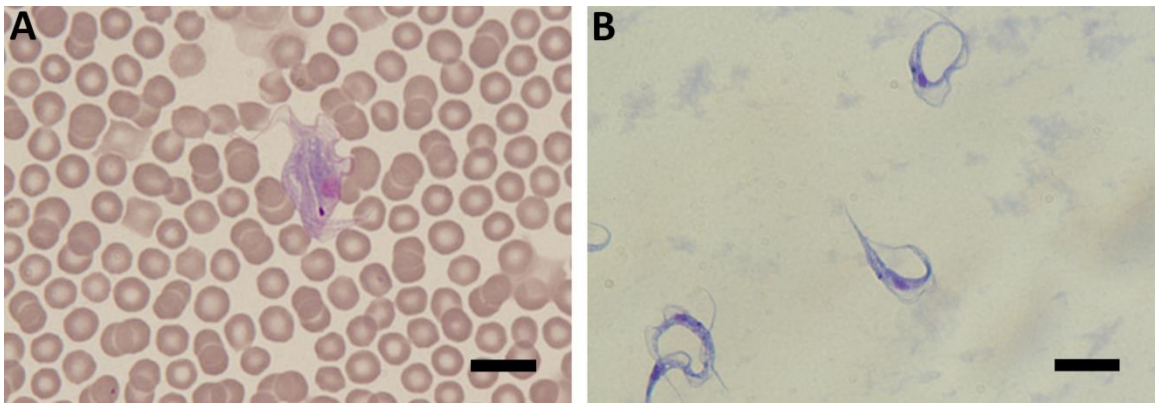


Figure 8. Light microscopy of Diff-quick stained *T. copemani*. A: trypomastigote forms in blood smears from woylies at the declining population; B: trypomastigote forms growing in culture at 37°C. Scale bars: 10 μm.

In the exponential or logarithmic phase of trypanosomes growing in culture, the epimastigote was the predominant form (Figure 9). Epimastigotes presented highly variable shapes with a small kinetoplast positioned adjacent to the nucleus; some of the forms were long and thin undergoing binary division, giving rise to nests or rosettes and transitional forms of variable shape and length of body. Some of these transitional forms were spheromastigotes that were often seen to be dividing (Figure 9). Figure 10 shows scanning electron microscopy images of an epimastigote and a spheromastigote from culture.

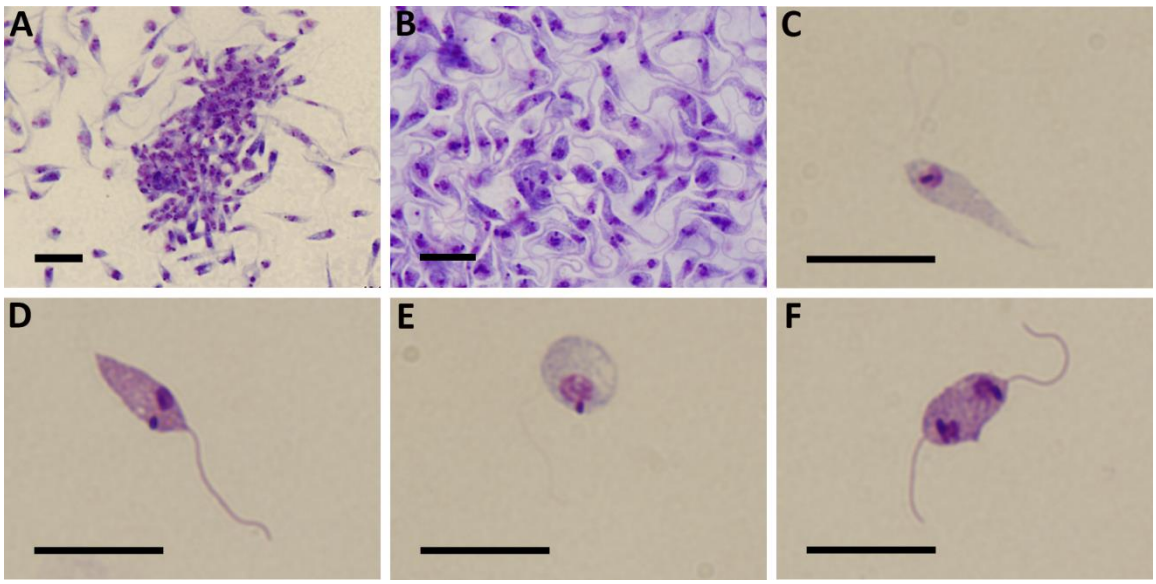


Figure 9. Light microscopy of exponential phase culture forms of *T. copemani* G1 and G2 strains (Clade A) and stained with Diff-Quick. A and B: nests of epimastigotes; C and D: epimastigotes with different shape; E: spheromastigote; F: spheromastigote dividing. Scale bars: 10 μm.

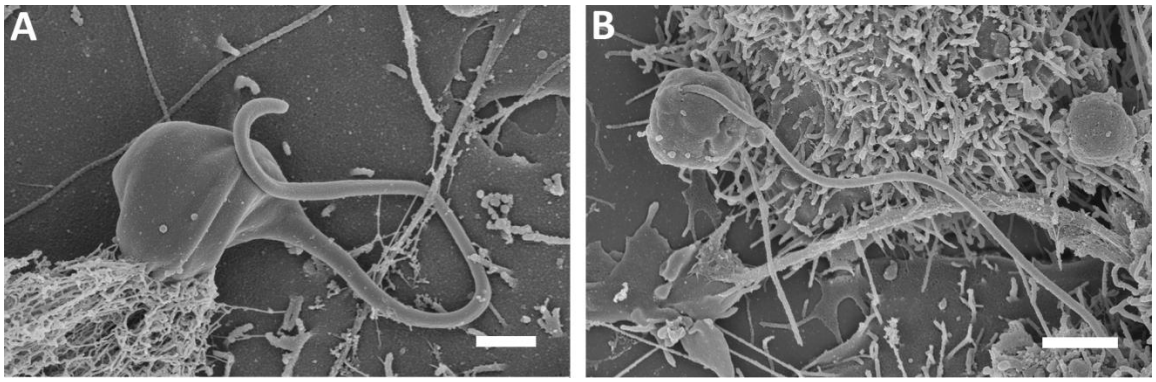


Figure 10. Scanning electron micrograph of *T. copemani* grown in culture. A: epimastigote; B: spheromastigote. Scale bars: 1 μm (A) and 2 μm (B).

Both strains reached the stationary or non-proliferative phase when the concentration of parasites was approximately $4x10^7$ parasites/ml (Figures 2 and 3). Most parasites at this late phase were slender and long flagellates that exhibited a rounded small kinetoplast positioned far away from an elongated nucleus and close to the posterior end of the cell resembling metacyclic trypomastigote forms (Figure 11). Interestingly, when trypomastigotes from the stationary phase were transferred from 28°C (the temperature used to grow epimastigotes in culture) to 37°C, an undulating membrane appeared again and trypanosomes morphology resembled the trypomastigote morphology observed in marsupial blood (Figure 8B). However, the size of the trypomastigote form in blood was notably bigger (Figure 8A).

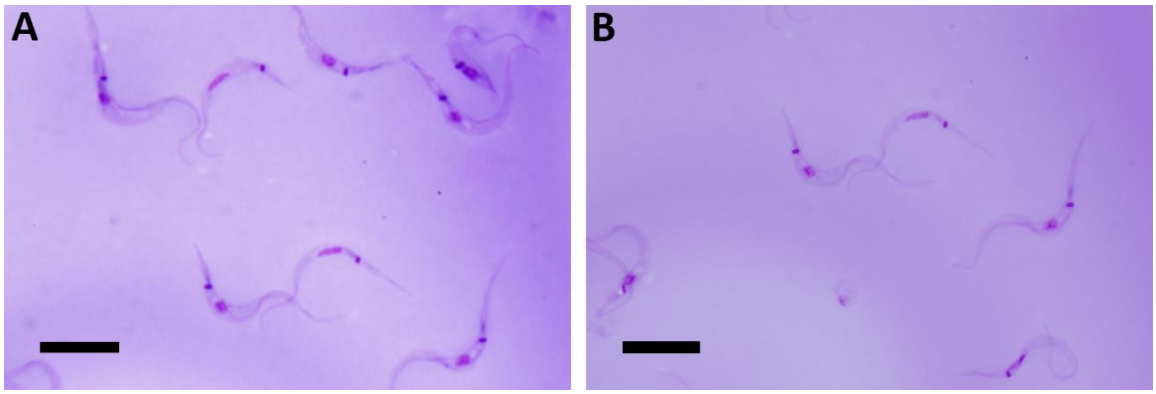


Figure 11. Light microscopy of stationary phase culture forms of *T. copemani* G1 and G2 strains (Clade A) and stained with Diff-Quick. A: *T. copemani* G1; B: *T. copemani* G2. Scale bars: 10 μm.

Analysis of the scanning and transmission electron microscopy images showed that *T. copemani* G1 and G2 shared morphological features with other trypanosomatids. Trypomastigotes, epimastigotes and spheromastigotes presented numerous spherical

electron-dense particles randomly distributed along the cytoplasm and morphologically very similar to acidocalcisomes described in other trypanosomatids. The size and number of acidocalcisomes per cell was variable ranging from only 3 or 4 big acidocalcisomes to more than 10 small acidocalcisomes (Figure 12). These structures were also observed in fresh smears by light microscopy (Figure 13). A dense corset of cross-linked microtubules that form the subpeculliar membrane was observed surrounding the body of the cell (Figures 12A and 14B).

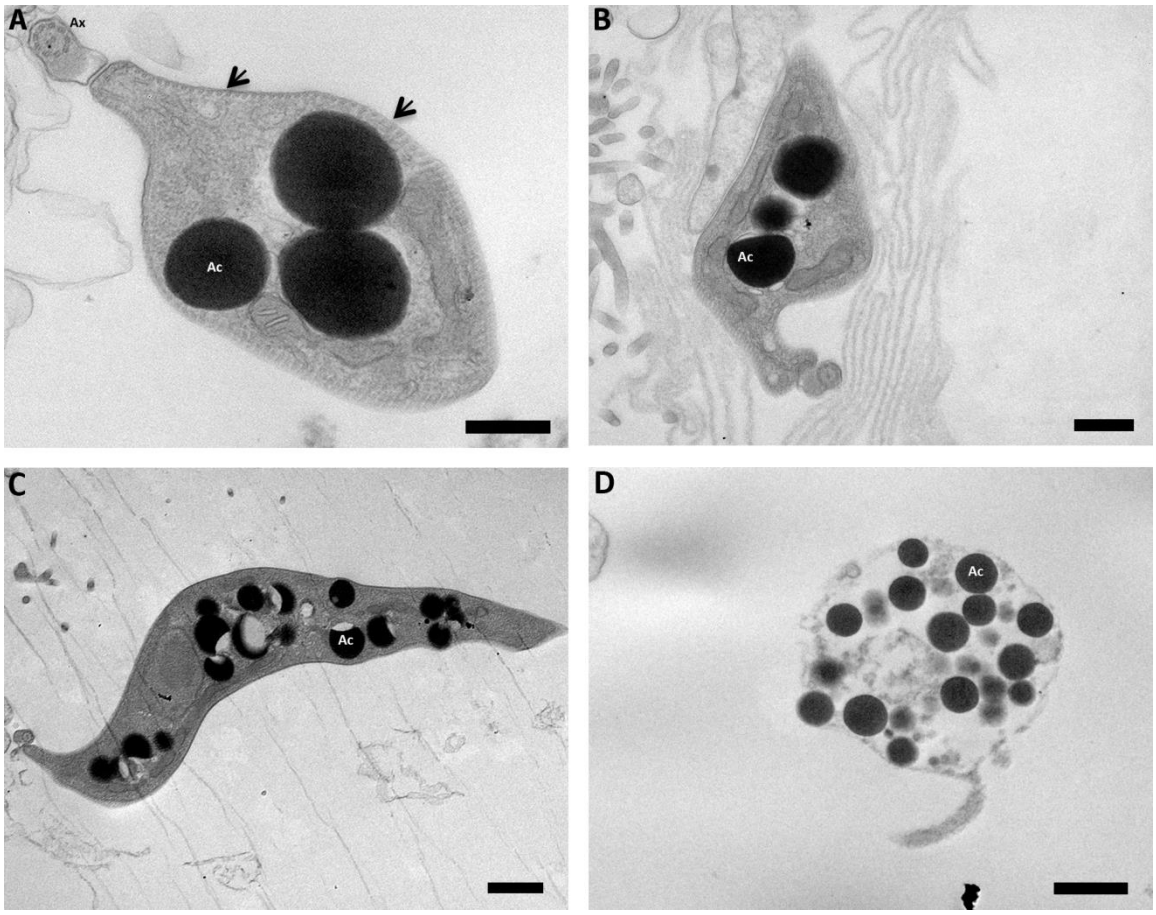


Figure 12. Transmission electron microscopy images of *T. copemani* acidocalcisomes. A and B: epimastigotes; C: trypomastigotes; D: spheromastigotes; Ac: acidocalcisomes; Ax: axoneme; Arrows: subpellicular microtubules (cytoskeleton). Scale bars: 0.5 μm (A and B), and 1 μm (C and D).

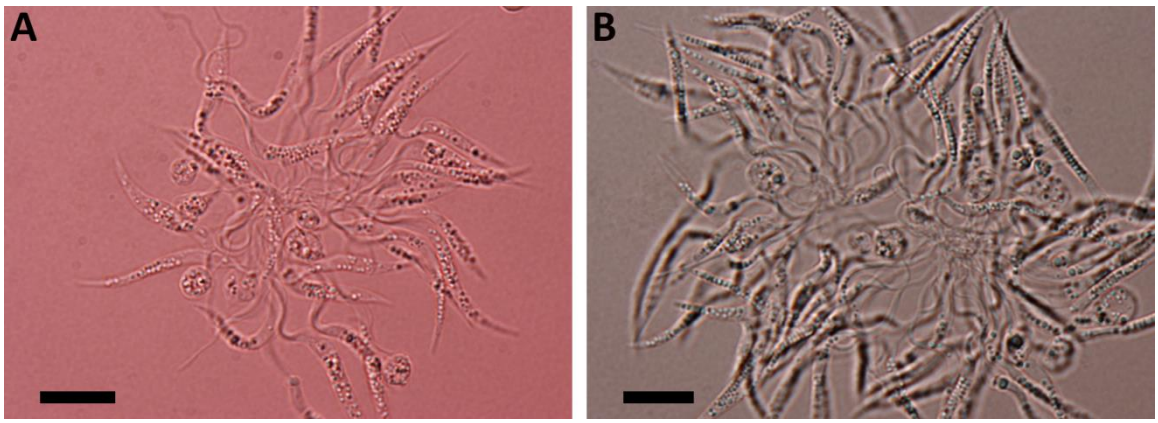


Figure 13. Light microscopy images of *T. copemani* with numerous acidocalcisomes (light green dots) in the cytoplasm. Scale bars: 10 μm.

Scanning electron microscopy images showed flagella that emerged from a small invagination in the cell body, the flagellar pocket. The flagellum of *T. copemani* consisted of an axoneme (AX) with nine duplets of microtubules in the periphery and two microtubules at the center, and the paraflagellar rod (PFR) (Figures 12A and 14).

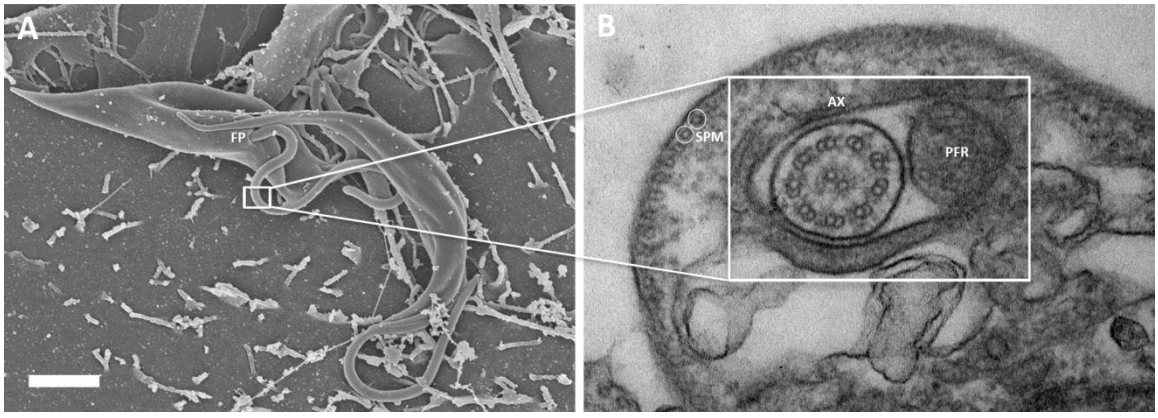


Figure 14. *T. copemani* epimastigotes and its flagellum structure. A: scanning electron microscopy image of epimastigotes B: transmission electron microscopy cross-section of an epimastigote. FP: flagellar pocket; AX: axoneme; PFR: paraflagellar rod; SPM: subpellicular microtubules. Scale bar: 2 μm.

4.5 Discussion

Although PCR results revealed infection with genotypes from Clade B trypanosomes in the majority of the blood samples from the stable population of woylies at Karakamia, no evidence of protozoa morphologically similar to *Trypanosoma* was observed in direct blood smears taken from these animals. Although a quantitative PCR was not used, PCR results from the 18S rDNA gene showed strong PCR products comparable with those obtained using DNA extracted from *T. copemani* growing in culture, possibly indicating high parasitemias with genotypes from this clade in the samples. A possible explanation for this finding is that the growth nutritional conditions that this species requires in culture were not supplied for the media used. However, the question remains as to why trypanosomes were not observed in any blood smears from samples that were positive by PCR. Interestingly, similar results were reported in previous studies. Smith *et al*. (2008) and Paparini *et al*. (2011) did not detect any trypomastigote forms in blood films, taken from woylies that were positive for different genotypes within Clade B by PCR and sequencing. Moreover, the description of *T. gilletti*, a *Trypanosoma* species closely related to Clade B trypanosomes, was based on molecular evidence alone, due to lack of definitive morphological measurements, since no trypomastigotes were observed in the blood smears from koalas infected with only this *Trypanosoma* species (McInnes *et al*. 2011). In an attempt to visualise and describe trypanosomes within this clade, a collaborative study was developed with Craig Thompson from Murdoch University. Smears of blood (previously found infected with trypanosomes from this clade by PCR) were processed by Fluorescence in situ Hybridization (FISH) using the Clade B species-specific forward primer (designed in Chapter 2) as a fluorescent probe. The results obtained revealed the presence of trypanosomes in the smears, much smaller than *T. copemani* (Clade A) and other

trypanosomes. This small *Trypanosoma* species is almost invisible under light microscopy demonstrating the difficulty in finding this parasite in smears of highly infected woylies. As a result of this investigation, we named all the genotypes belonging to this clade "*T. vegrandis*", which was published in the Journal "Parasites and Vectors" (See attached paper in the Appendix II) (Thompson *et al*. 2013).

Although hemin was not necessary for the growth of *T. copemani in vitro*, it enhanced cell proliferation considerably. A haemin concentration of 2.5 mg/l added to Grace's medium was the optimum concentration for the growth of both genotypes of *T. copemani* in culture. Haemin concentrations higher than 15 mg/l produced markedly significant morphological changes. Most of the forms present in these cultures were spherical with a short or no flagella consistent with amastigote forms of other *Trypanosoma* species, suggesting that transformation from epimastigotes to amastigotes could be induced by high concentrations of haemin. These results are in agreement with previous studies that showed that differentiation of *T. cruzi* epimastigotes to amastigotes could be achieved by high concentrations of haemin in the media used (Ciccarelli *et al*. 2007). Pal *et al*. (2001) also showed that high concentrations of haemin in cultures of *Leishmania donovani* triggered promastigote-amastigote transformation.

The differentiation of epimastigotes to metacyclic infective trypomastigotes could be triggered by nutritional stress. It has been shown that the change of *T. cruzi* epimastigotes from a nutrient rich medium to a nutrient poor medium resulted in differentiation to metacyclic trypomastigotes (Figueiredo *et al*. 2000). Furthermore, *T. cruzi* metacyclogenesis has been shown to occur in cultures where the medium has not been replaced with fresh medium (Camargo. 1964, Figueiredo *et al*. 2000). The

results of this study are consistent with these previous studies and show metacyclogenesis of *T. copemani* is induced by nutrients and growth factor limitations.

T. copemani nests moved across the plate surface, recruiting neighbouring smaller nests establishing large communities of cells similar to what have been described in bacteria and what is termed "social motility" (Velicer *et al*. 2000). Several studies have demonstrated cooperative movements or social motility among bacteria in the surface of semisolid medium in response to external signals (Harshey. 2003). It has also been shown that *T. brucei* engages in social behaviour when cultivated on semisolid agarose surfaces. Oberholzer *et al*. (2010) showed in *in vitro* experiments that *T. brucei* is able to assemble into multicellular communities with polarised and coordinated movements that are not apparent in single cells and are in response to an external stimulus. It has been suggested that the social motility in *T. brucei* may facilitate colonisation of host tissues and may have impact in pathogenesis. In the mammalian host for example, *T. brucei* penetration of the blood brain barrier represents a critical and defining step of disease pathogenesis, and social motility might be enhancing it (Velicer *et al*. 2000). In bacteria, social motility provides many advantages, such as enhanced tissue colonisation, and increased resistance to host defense mechanisms (Bassler *et al*. 2006, Zusman *et al*. 2007). The apparent social motility observed in *T. copemani* could have some important consequences in infectivity and pathogenicity and might enhance their ability to colonise, penetrate and migrate through different tissues in the vertebrate and invertebrate host.

T. copemani displayed morphological features including ultrastructural organisation which is consistent with other trypanosomatids. Acidocalcisomes are rounded electron-

dense acidic organelles, rich in calcium, that have been reported in different trypanosomatids such us *T. cruzi* (Docampo *et al*. 1996, Miranda *et al*. 2000), *Leishmania amazonensis* (Lu *et al*. 1997), *T. erneyi* (Lima *et al*. 2012), *T. evansi* (Mendoza *et al*. 2002), *T. brucei* (Scott *et al*. 1995). The finding of numerous acidocalcisomes in the cytoplasm of *T. copemani* may be relevant from the point of view of infectivity. It has been demonstrated that these structures play an important role in host cell invasion and in the intracellular development of trypanosomes. In *Trypanosoma cruzi* for example, an increase in the acidocalcisome Ca^{2}+ concentration of trypomastigotes occurs upon invasion, and pretreatment of trypomastigotes with intracellular Ca^{2}+ chelators prevented the increase of acidocalcisome Ca^{2}+ and resulted in an inhibition of cellular invasion (Docampo, and Moreno. 1996, Moreno *et al*. 1994, Yakubu *et al*. 1994). A morphometric study in different trypanosomatids showed that "acidocalcisomes are often large when present in low numbers and small when there are many present" (Miranda *et al*. 2004). Although the number and size of *T. copemani* acidocalcisomes is variable, the size of the organelle seems to be inversely proportional to the number of organelles present. When acidocalcisomes were large in size, the number of these structures in each cell was no more than three. In contrast, when the size of the acidocalcisomes was small, more than ten structures were found in each cell. The significance of the presence and abundance of acidocalcisomes in *T. copemani* needs to be further investigated especially from the point of view of infectivity.

Another important organelle present in most trypanosomes is the flagellar pocket. This organelle is a deep invagination of the membrane at the site where the flagellum exits the cytoplasm. The flagellar pocket is the unique site of trypanosome exocytosis and endocytosis, and together with the flagella is involved in cell division (Field *et al*. 2009). The flagellum of *T. copemani* consists of the axoneme (AX) and the paraflagellar rod

(PFR). The axoneme, similar to other trypanosomes, presented nine duplets of microtubules, and the PFR was situated adjacent to the axoneme. Although the ultrastructure of the PFR appears conserved throughout Kinetoplastids, the PFR ultrastructure differs in size at the interspecies level and in some cases a significantly reduced PFR is present (Bastin *et al*. 1996, Maga *et al*. 1999, Portman *et al*. 2010). Some trypanosomatids such as *Crithidia deanei, C. oncopelti, Blastocrithidia culicis* and *Herpetomonas roitmani* have been described as lacking a PFR (Freymuller *et al*. 1981). Although *T. copemani* possess a PFR, its internal structure needs to be investigated.

In summary, this study describes the isolation and growth requirements of two different strains of *T. copemani* from the woylie, and highlights important morphological features shared between this parasite and other pathogenic trypanosomes.

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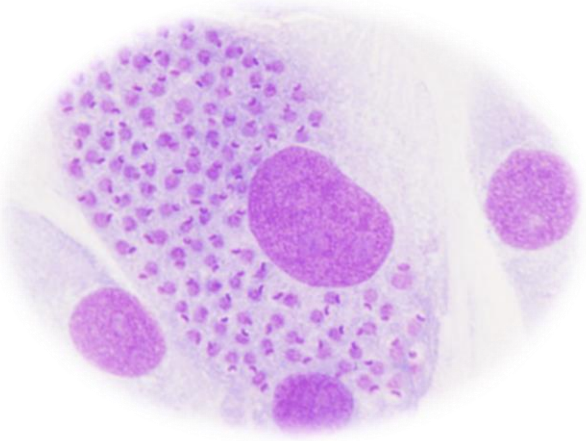
Chapter 5

Infection kinetics of two strains of Trypanosoma copemani: a comparative in vitro study with the pathogenic Trypanosoma cruzi



5.1 Abstract

The *in vitro* kinetics of infection of two strains of *T. copemani* and one strain of *T. cruzi* was investigated and compared in four different cell lines: the non-phagocytic *L6*, *VERO* and *HCT8* cells, and the phagocytic human derived cell line *THP1*. Results showed that both strains of *T. copemani* were able to infect cells. However, significant differences in infection capability and intracellular replication were seen between them and in comparison to *T. cruzi*. The rate of infection of *T. copemani* G1 and G2 in most cell lines was always below 7% and 15% respectively. However, *T. copemani* G2 rate of infection in *VERO* cells was considerably higher, with 70% of cells infected at 48 hours post-infection. *T. cruzi* was highly infective to all cell lines, with *L6* and *VERO* cells presenting the highest (82%) and lowest (33%) percentage of infection respectively. Despite the higher rate of infection of *T. copemani* G2 in *VERO* cells, when compared with *T. cruzi,* the number of intracellular forms was lower. The capability of *T. copemani* to infect cells *in vitro* and the findings of structures suggestive of intracellular amastigotes in woylie tissues infected with *T. copemani* G2 (Chapter 3) suggests that this parasite might employ similar strategies to complete its life cycle in the vertebrate host to those seen in *T. cruzi*. The capability of *T. copemani* to infect cells may have important consequences for pathogenicity.

5.2 Background

Trypanosoma comprises a large number of species and subspecies with a complex life cycle that involves both invertebrate and vertebrate hosts (Teixeira *et al*. 2012). The life cycle in the vertebrate host varies from one species to another and may involve different stages of the parasite. This variability mainly lies in the ability of some trypanosomes to migrate to tissues and to replicate inside host cells.

The replication of trypanosomes such us *T. theileri,* a parasite of cattle, *T. lewisi* a parasite of rodents, and trypanosomes from the *T. brucei* complex that infect humans and several ungulates, occurs extracellularly in peripheral blood, where they replicate as trypomastigotes. This stage of the parasite has also been found in extravascular sites of lymph nodes, kidney, spleen, bone marrow, and brain (Hoare. 1972, Molyneux. 1976, Tizard *et al*. 1980, Sudarto *et al*. 1990, D'Alessandro *et al*. 1991, Rodrigues *et al*. 2003). In contrast, *T. cruzi*, the agent of Chagas disease does not replicate in blood, but presents a life cycle that involves invasion and replication inside host cells (Oliveira *et al*. 2009, De Souza *et al*. 2010). During its life cycle in the vertebrate host, *T. cruzi* alternates between two different developmental stages, the bloodstream trypomastigote (non-dividing form), and the amastigote in tissues (intracellular and replicative form). Bloodstream trypomastigotes invade a large number of mammalian cells, and once in the cytoplasm of the cell they differentiate into amastigotes. After an intense multiplicative phase, amastigotes transform into trypomastigotes that are released upon rupture of the cells. Trypomastigotes infect neighbouring cells, and are eventually disseminated throughout the body, leading to the establishment of the infection in several tissues in the host (De Souza *et al*. 2010). Although *T. cruzi* can infect several tissues in the vertebrate host, such as spleen, liver, colon, skeletal muscle, and heart, it

has been demonstrated that different populations of the parasite present distinct tissue preferences or "tissue tropism" (Andrade *et al*. 1999). Studies have shown a correlation between *in vitro* and *in vivo* tissue tropism using two different strains of *T. cruzi*. Andrade *et al*. (2002) showed that the *T. cruzi* strain JG was more predominant in the hearts of experimentally chronically infected BALB/c and DBA-2 mice than the *T. cruzi* strain Col1.7G2. In a more recent study, the same authors showed that in *in vitro* studies using cardiomyocytes cells, the JG strain exhibited a higher intracellular multiplication than the Col1.7G2 strain (Andrade *et al*. 2010).

A few other *Trypanosoma* species are capable of infecting and multiplying within cells. For example, *T. dionsii*, *T. erneyi*, and *T. livingstonei*, all trypanosomes of bats, are capable of invading and replicating inside cells and have been shown to exhibit some similar features of host cell invasion with *T. cruzi* (Oliveira *et al*. 2009, Lima *et al*. 2012, Lima *et al*. 2013). For *T. rangeli*, a parasite that infects a large number of mammals in Central and South America, contradictory results have been reported regarding the life cycle within the vertebrate host. The replication of this species does not occur in blood, however, its ability to infect and replicate inside cells has been debated with some studies showing that *T. rangeli* is able to infect cells, whilst others show the opposite (Zúñiga *et al*. 1997a, Zuñiga *et al*. 1997b, Tanoura *et al*. 1999, Eger-Mangrich *et al*. 2001).

Within Australia, several species of *Trypanosoma* have been described infecting wildlife. These include *T. thylacis* in northern brown bandicoots (*Isoodon macrourus*) (Mackerras. 1959); *T. lewisi* from the chuditch (*Dasyurus geoffroii*), ash-grey Mouse (*Pseudomys albocinereus*), bush rat (*Rattus fuscipes*), and the dibbler (*Parantechinus*

apicalis) (Averis *et al*. 2009); *T. irwini* and *T. gilletti* in koalas (*Phascolarctos cinereus*) (McInnes *et al*. 2009, 2011b); *T. binneyi* in platypus (*Ornithorhynchus anatinus*) (Noyes *et al*. 1999); *T.* sp H25 in eastern Grey Kangaroos (*Macropus giganteus*), woylies (*Bettongia penicillata*), banded hare wallabies (*Lagostrophus fasciatus*) and boodies (*Bettongia lesueur*) (Chapter 2) (Botero *et al*. 2013); *T.* sp H26 in the common wombat (*Vombatus ursinus*); *T. vegrandis* in woylies (*Bettongia penicillata*), tammar wallabies (*Macropus eugenii*), the chuditch (*Dasyurus geoffroii*), and western grey kangaroos (*Macropus fuliginosus*) (Chapter 2) (Botero *et al*. 2013, Thompson *et al*. 2013); and *T. copemani* in quokkas (*Setonix brachyurus*), Gilbert's potoroo (*Potorous gilbertii*) (Austen *et al*. 2009), koalas (*Phascolarctos cinereus*) (McInnes *et al*. 2011a, 2011b), woylies (*Bettongia penicillata*), common brush-tailed possums (*Trichosurus vulpecula*), the quokka (*Setonix brachyurus*), the chuditch (*Dasyurus geoffroii*), and southern brown bandicoots (*Isoodon obesulus*) (Chapter 2) (Botero *et al*. 2013, Thompson *et al*. 2013). Despite such an extensive list, only one study has provided insights into the life cycle of Australian trypanosomes in the vertebrate host. Noyes *et al.* (1999) showed that *T.* sp H25 isolated from a kangaroo, and *T.* sp H26 isolated from a wombat were not able to infect *LLCMK2* cells *in vitro* or in immunocompetent mice *in vivo*.

Considering that *T. copemani* is able to migrate to different organs in the marsupial host and has the ability of producing pathogenic lesions in tissues, the aims of this study were to: (i) investigate the capability of *T. copemani* to infect cells *in vitro*; and (ii) compare the infection kinetics of *T. copemani* and the pathogenic *T. cruzi* in four different cell lines.

5.3 Materials and methods

5.3.1 Cells and parasites

The non-phagocytic cell lines *L6* (skeletal myoblast cells), *VERO* (kidney epithelial cells), and *HCT8* (human ileocecal adenocarcinoma cells) and a phagocytic cell line (macrophage-like cells) derived from the human monocyte cell line *THP1* were used. Cells were grown in RPMI medium supplemented with 10% foetal calf serum (FCS) at 37°C and 5% CO_2. *THP1* cells were grown in media that also included phorbol 12-myristate 13-acetate (PMA). *T. copemani* G1 and G2 and the *T. cruzi* strain 10R26 were maintained in RPMI medium containing 10% FCS plus 5mg/ml penicillin-streptomycin. All cell lines and trypanosomes were stored in a cryobank at Murdoch University.

5.3.2 Cell infection

Monolayers of each cell line were trypsinised and seeded onto tissue culture-slides (16-wells) at a concentration of 1.5×10^3 cells/ml. Two sets of six culture-slides were used. The first set contained *L6* and *VERO* cells and the second set contained *THP1* and *HCT8* cells. After 24 hours, the media was discarded to remove non-adherent cells and 100 µl of parasite suspension containing 1.5×10^5 trypanosomes/ml was added to each well (1:10 cell/parasite ratio). Cultures from the stationary phase containing metacyclic trypomastigote forms of *T. copemani* G1 and G2, and *T. cruzi* were used to infect cells. Slides were incubated at 37°C and 5% CO_2. Experiments were replicated on three separate occasions.

5.3.3 Kinetics of infection

The progress of cellular infection was monitored at 3, 6, 12, 24, 48, 72, and 96 hours post-infection. Non-adherent parasites were removed by washing the culture-slides three times with phosphate buffer solution (PBS) at each interval of time. Coverslips were removed and culture-slides were air-dried and stained with a Diff-Quik staining system. The percentage of infected cells was determined under 100x magnification by counting 100 cells/well per triplicate using an optical microscope and comparing the number of cells containing intracellular parasites to the total number of cells.

5.3.4 Scanning and Transmission Electron Microscopy

For scanning electron microscopy (SEM) cells were pre-seeded on poly-L-lysine coated coverslips in 24-well plates (2×10^5 cells/well) and infected with *T. copemani* G1 and G2 as above. After 48 hours post-infection, the coverslips were removed, washed in PBS, and fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS : cell culture media (pH 7.2). Coverslips were subsequently progressively dehydrated through a graded series of ethanols, using a PELCO Biowave microwave and critical point dried as previously described (Edwards *et al*. 2011). Coverslips were mounted on stubs with adhesive carbon, coated with 2 nm Pt and imaged at 3 kV using the in-lens secondary electron detector on a Zeiss 55VP field emission SEM.

For transmission electron microscopy (TEM), infected cells were trypsinised at 48 hours post-infection and fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS : cell culture media (pH 7.2). Cells were pelleted, with all subsequent processing performed in a PELCO Biowave microwave. Briefly, samples were post-fixed in 1% OsO_4 in PBS

followed by progressive dehydration in a graded series of ethanol/acetone, before being infiltrated and embedded in the epoxy resin Procure-Araldite. Sections 120 nm-thick were cut on a diamond knife and mounted on copper grids. Digital images were collected from unstained sections at 120 kV using a JEOL 2100 TEM fitted with a Gatan ORIUS1000 camera.

5.3.5 Statistical analyses

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, California, USA). Analysis of the data was performed using one-way and two-way analysis of variance (ANOVA). The results were expressed as means ± SEM.

5.4 Results

To help elucidate the life cycle of *T. copemani*, four different cell lines were infected with two strains of *T. copemani* (G1 and G2) and with one strain of the intracellular *T. cruzi,* which was used as a control of cell infection and a point of comparison. Stationary phase culture smears from all the strains were stained and checked under the microscope to confirm that metacyclic trypomastigotes were used to infect cells. Results showed both strains of *T. copemani* were able to infect all cell lines used. However, significant differences in the infection rate were seen between *T. copemani* G1 and G2, and between both *T. copemani* G1/G2 and *T. cruzi* in all cell lines ($p<0.0001$). No signs of intracellular replication were observed in the cytoplasm of any cell line infected with either *T. copemani* G1 or G2. Therefore, the results regarding the number of amastigotes inside cells were not quantified and are expressed as qualitative observations.

5.4.1 Kinetics of infection in L6 cells

Intracellular amastigotes of *T. copemani* G1 and G2 and *T. cruzi* were observed for the first time at 6 hours post-infection. The progression of the infection was similar for *T. copemani* G1 and G2. However, the number of cells infected with *T. copemani* G2 was significantly higher ($P<0.0001$). The highest rate of infection by *T. copemani* G1 was 5% at 48 hours post-infection, while the highest rate of infection by *T. copemani* G2 was 15% at the same time post-infection. In contrast, the percentage of *L6* cells infected with *T. cruzi* was significantly higher ($p<0.0001$), increasing rapidly after 24 hours post-infection and reaching a peak of 82% at 72 hours post-infection (Figure 1). Cells infected with *T. cruzi* exhibited a larger number of intracellular amastigotes at 12, 24, 48, 72 and 96 hours post-infection compared with both *T. copemani* G1 and G2, where no more than three amastigotes were seen inside cells at any time post-infection (Figure 1 - light micrographs). After 48 hours post-infection, *T. cruzi* amastigotes were seen differentiating into trypomastigotes inside cells (Figure 2).

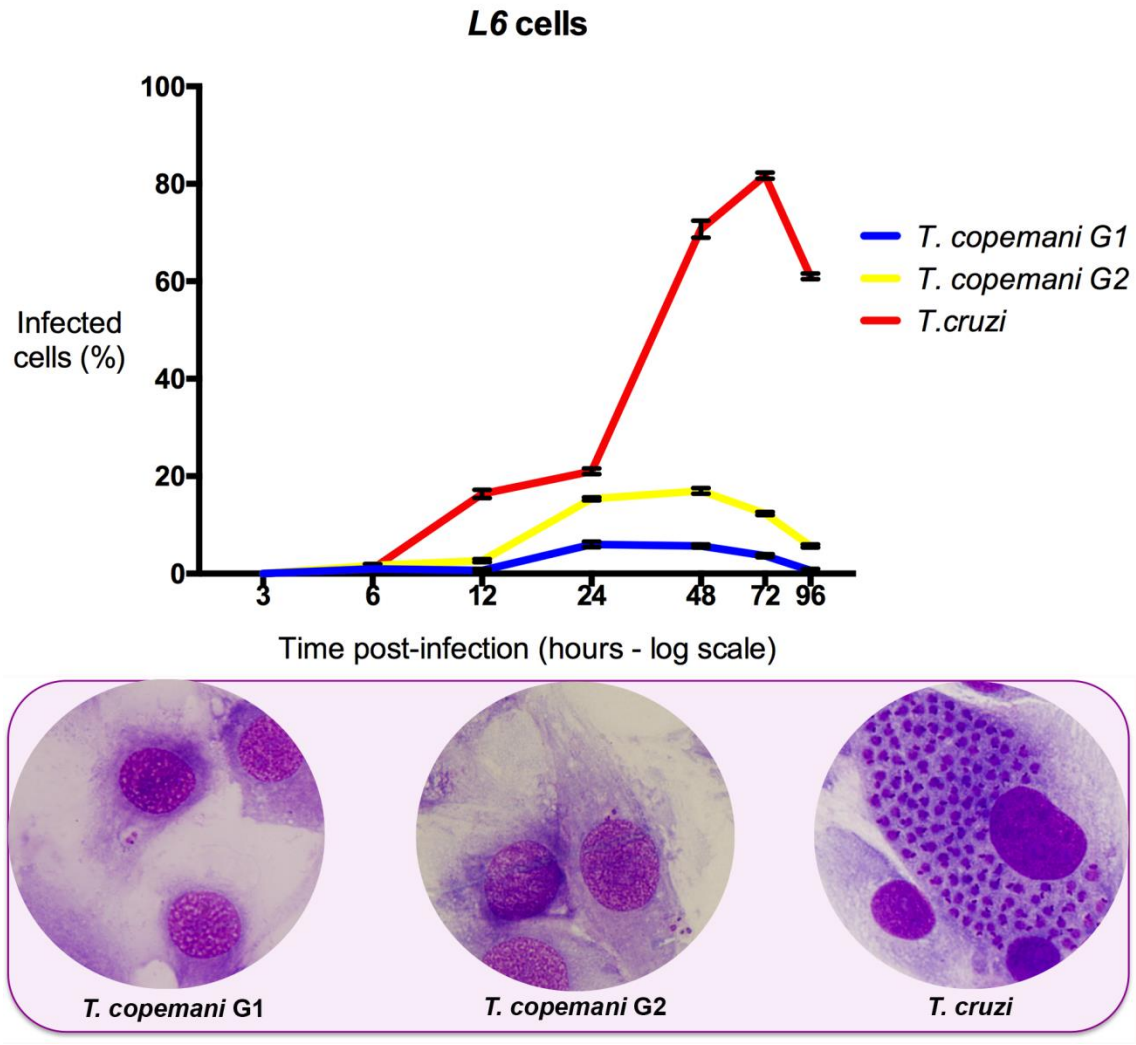


Figure 1. Kinetics of infection and intracellular development in *L6* cells infected with *T. copemani* G1 and G2, and *T. cruzi*. The data represents the mean of three independent experiments (triplicate values in each experiment) ± SEM.

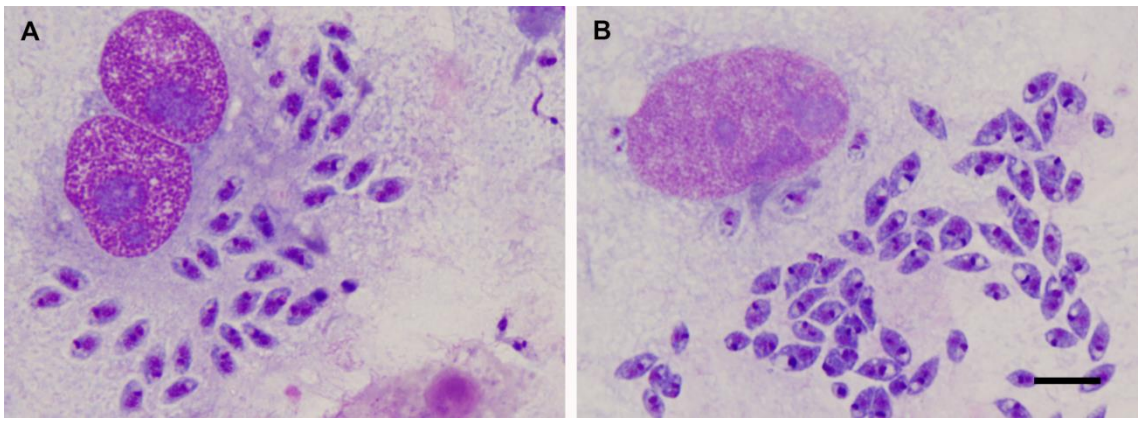


Figure 2. *T. cruzi* amastigotes differentiating into trypomastigotes. Scale bar: 10 μm.

5.4.2 Kinetics of infection in VERO cells

In general, both *T. copemani* G1 and G2, and *T. cruzi* were able to infect *VERO* cells at 6 hours post-infection, with no significant differences in the number of cells infected between them ($p<0.07$). An increase in the number of cells infected was observed over the time, reaching a maximum of 7% with *T. copemani* G1, 74% with *T. copemani* G2, and 33% with *T. cruzi* at 48 hours post-infection. After this time, the percentage of infected cells decreased for all trypanosomes (Figure 3). *T. copemani* G2 induced a significant stronger infection at all times post-infection when compared with *T. cruzi* ($p<0.0001$). Although almost 70% of cells were infected with *T. copemani* G2 and only 33% of cells were infected with *T. cruzi* at 48 hours post-infection, the number of intracellular amastigotes was higher in *T. cruzi* infected cells (Figure 3 - light micrographs).

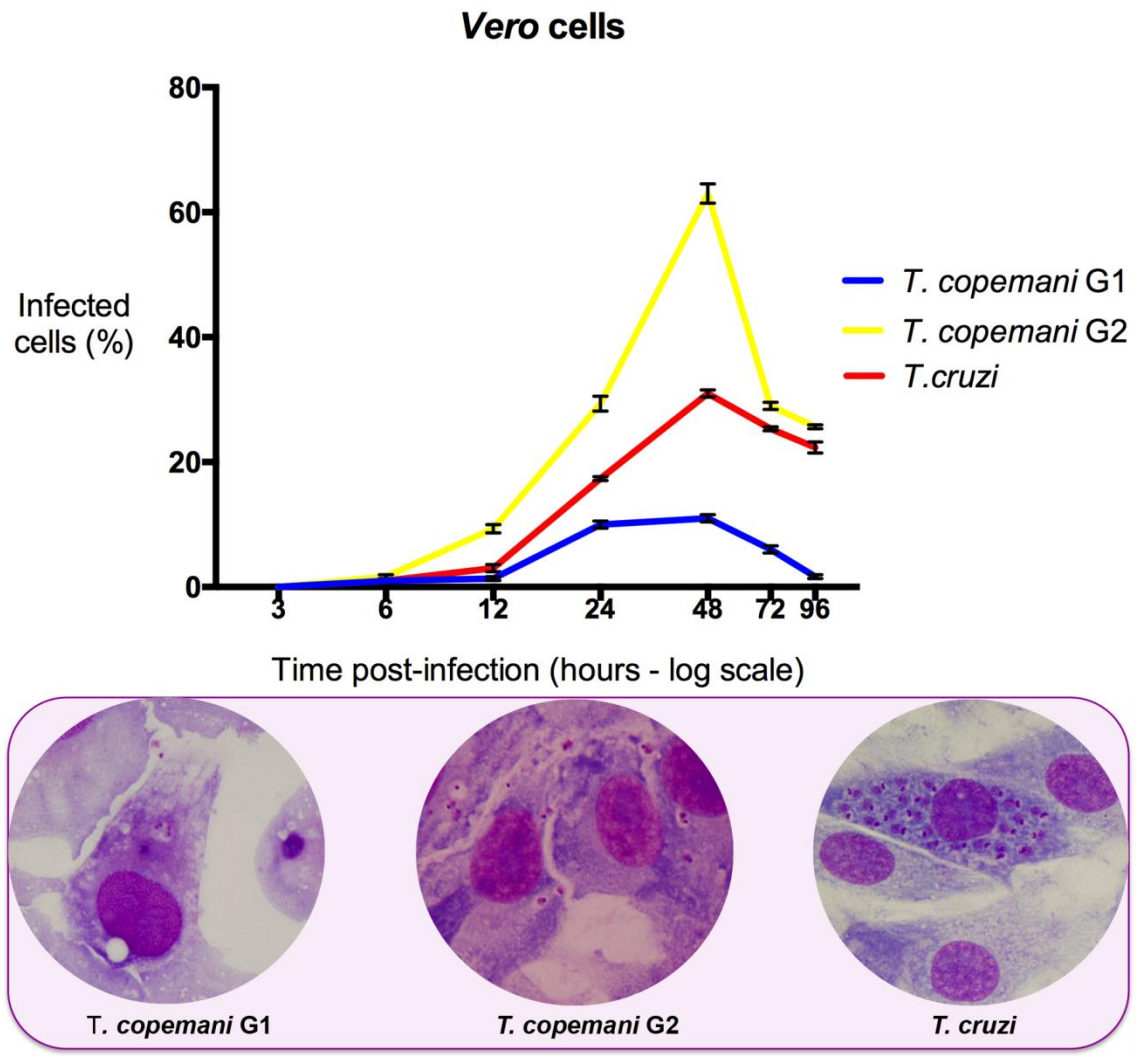


Figure 3. Kinetics of infection and intracellular development in *VERO* cells infected with *T. copemani* G1 and G2, and *T. cruzi*. The data represents the mean of three independent experiments (triplicate values in each experiment) ± SEM.

5.4.3 Kinetics of infection in HCT8 and THP1 cells

The rate of infection of each *Trypanosoma* strain was very similar in both human derived cell lines *HCT8* and *THP1* (Figure 4). Neither *T. copemani* G1 or G2 were able to infect more than 7% of either cell line at any time post-infection. In contrast, the percentage of cell infected with *T. cruzi* was significantly higher ($p<0.0001$), and

showed a continual increase in infection rates, which peaked at 34% in *HCT8* cells and 40% in *THP1* cells at 96 hours post-infection. Active intracellular replication was clearly evident only in *T. cruzi* where large numbers of amastigotes were seen inside cells (Figure 4 - light micrographs).

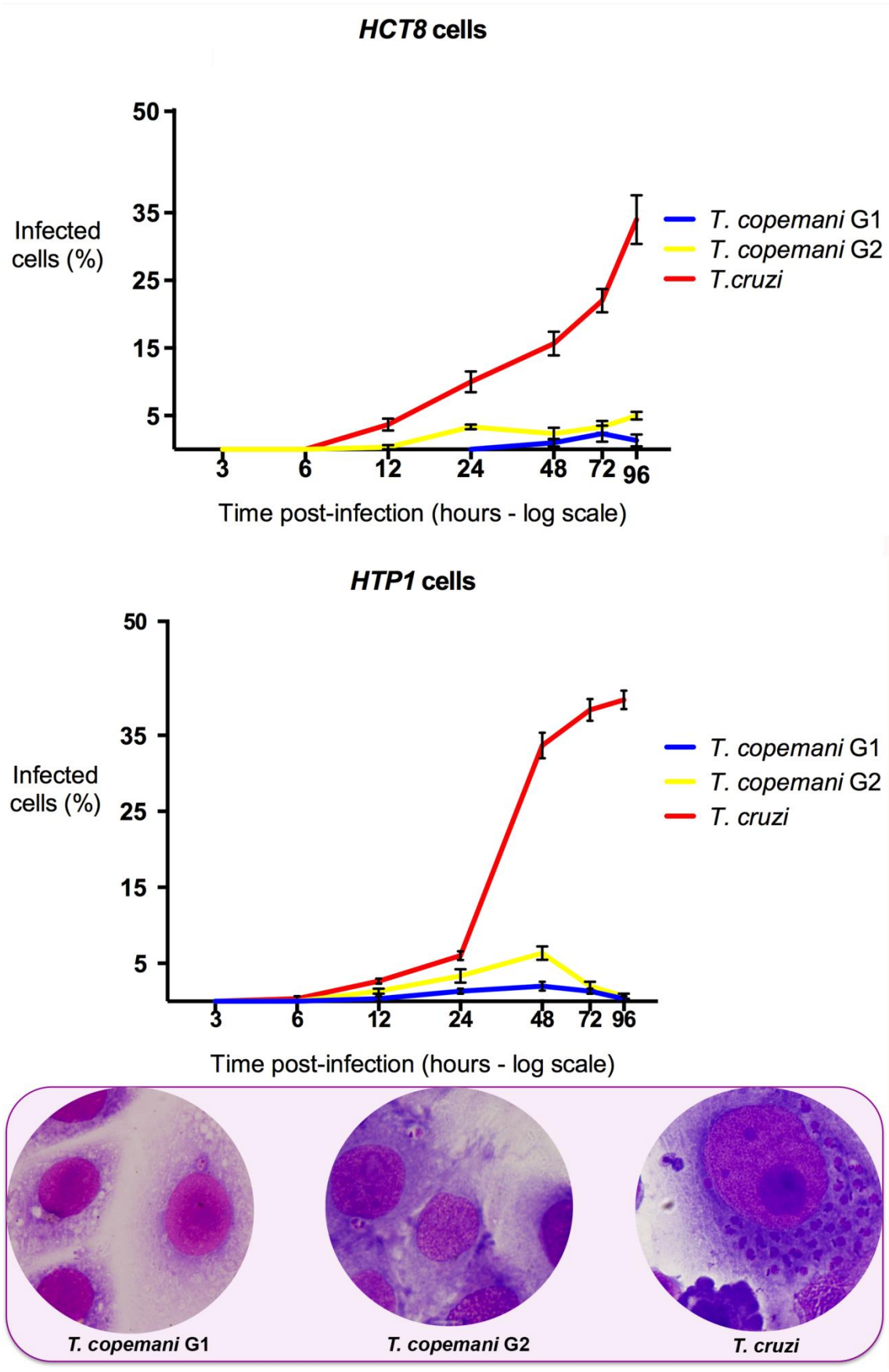

HCT8 cells
Infected cells (%)
50
35
25
15
5
3
6
12
24
48
72
96
Time post-infection (hours - log scale)
T. copemani G1
T. copemani G2
T.cruzi
HTP1 cells
Infected cells (%)
50
35
25
15
5
3
6
12
24
48
72
96
Time post-infection (hours - log scale)
T. copemani G1
T. copemani G2
T. cruzi
T. copemani G1
T. copemani G2
T. cruzi

Figure 4. Kinetics of infection and intracellular replication in *HCT8* and *THP1* cells infected with *T. copemani* G1, *T. copemani* G2 and *T. cruzi*. The data represents the mean of three independent experiments (triplicate values in each experiment) ± SEM.

5.4.4 Transmission and scanning electron microscopy

Trypomastigotes were commonly observed attached to cells by light microscopy (Figure 5). They appeared to attach to the cell by the posterior end in a manner similar to *T. cruzi*.

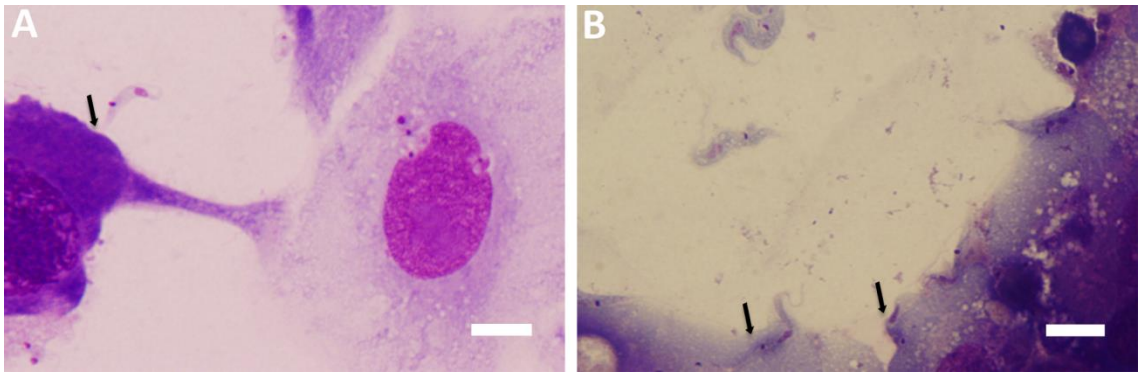


Figure 5. Trypomastigotes of *T. copemani* G2 attached and penetrating *VERO* cells. A: light micrograph of a trypomastigote attached to a *VERO* cell; B: light micrograph of trypomastigotes penetrating *VERO* cells. Arrows: trypomastigotes. Scale bars: 10 μm.

Scanning electron microscopy further confirmed that attached trypanosomes were invading cells and commonly revealed debris of dead cells surrounded by amastigotes and trypomastigotes after the second day of infection (Figure 6). The presence of free amastigotes in proximity to dead cells could indicate that they were released after the death of the cell.

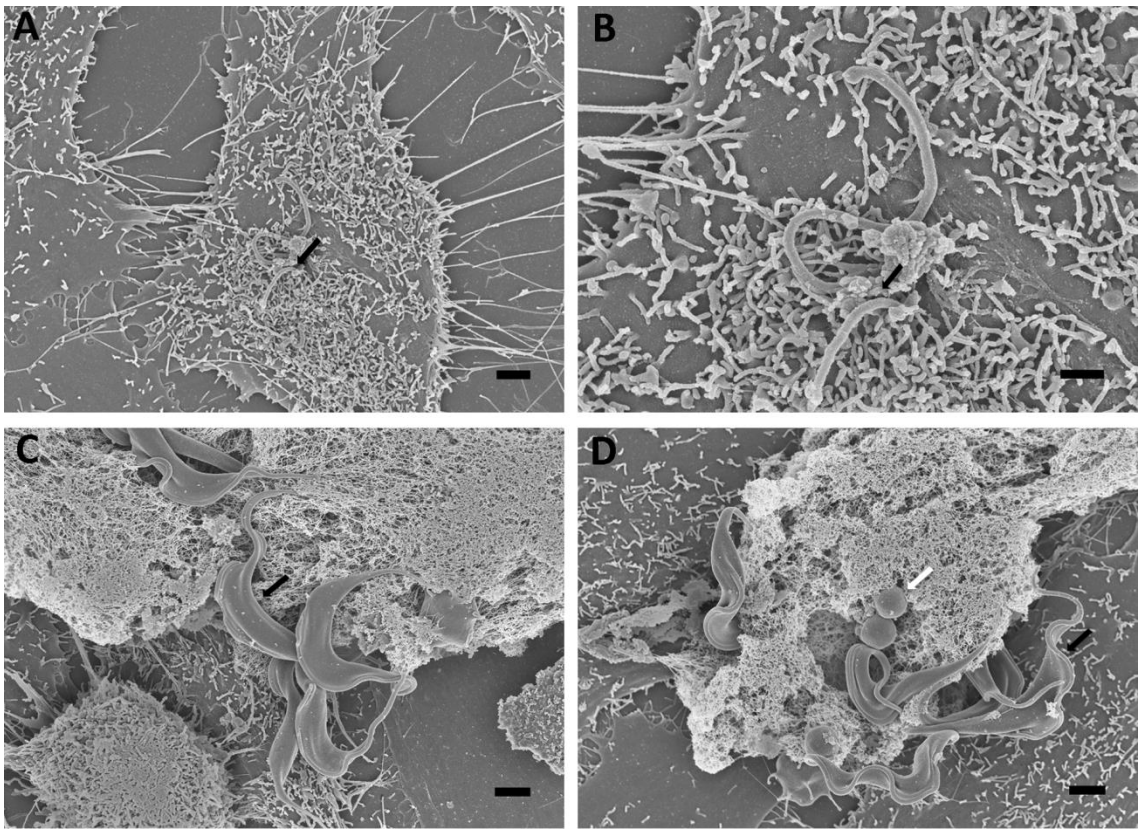


Figure 6. Scanning electron micrographs of trypomastigotes and amastigotes of *T. copemani* G2 in *VERO* cells. A and B: trypomastigotes invading a cell; B: trypomastigotes and amastigotes in cellular debris. Black arrows: trypomastigotes. White arrow: amastigotes. Scale bars A, C and D: 2 μm. Scale bar B: 1 μm.

Analysis of thin sections by TEM confirmed that *T. copemani* is able to attach (Figures 7A and B), and invade cells (Figures 8A and B). Intracellular *T. copemani* G2 amastigotes containing numerous acidocalcisomes were identified within the cell cytoplasm (Figures 8 A and B).

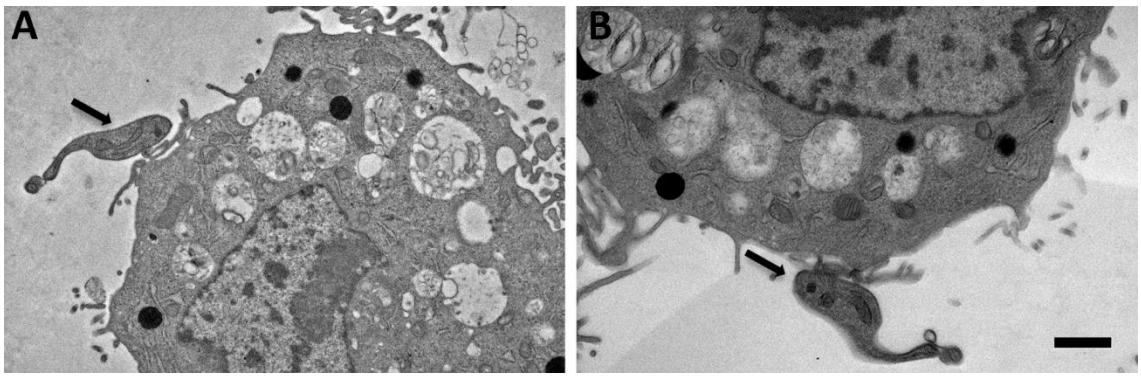


Figure 7. Transmission electron micrographs of trypomastigotes of *T. copemani* G2 attached to cells. Arrows: trypomastigotes. Scale bar: 1 μm.

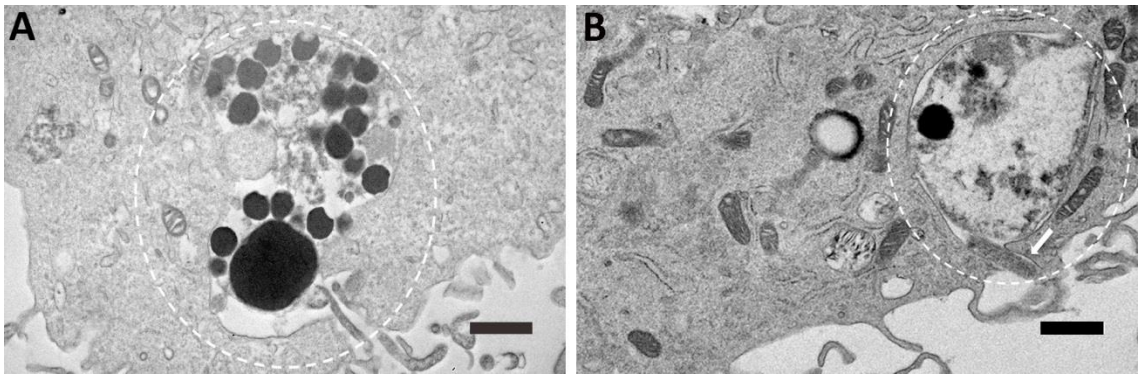


Figure 8. Transmission electron micrographs of intracellular amastigotes of *T. copemani* G2. Circles: intracellular amastigotes. Arrow: remaining flagella. Scale bars: 1 μm.

5.5 Discussion

A few species of *Trypanosoma* have been isolated in culture from Australian mammals (Noyes *et al*. 1999, Austen *et al*. 2009). However, the biological life cycle of these parasites in the vertebrate host remains unknown. To date, the only *in vitro* study investigating the life cycle of Australian trypanosomes showed that two *Trypanosoma* species, *T.* sp H25 and *T.* sp H26 isolated from a kangaroo and a wombat respectively,

failed to infect *LLCMK2* cells (Rhesus monkey kidney cells) (Noyes *et al*. 1999). As previously demonstrated in Chapters 2 and 3, *T. copemani* is able to colonise different tissues in naturally infected marsupials. Findings of DNA of *T. copemani* in tissues and structures in the cytoplasm of these tissue cells consistent with amastigote forms of other *Trypanosoma* species (Chapters 2 and 3) (Carreira *et al*. 1996, Botero *et al*. 2013), suggested a *T. copemani* life cycle in the marsupial host that might involve infection of cells and intracellular replication.

In attempts to demonstrate this, this study investigated the *in vitro* capability and progress of infection of two different strains of *T. copemani* (G1 and G2) in four different cell lines and compared it with the progress of infection of the well-known intracellular parasite *T. cruzi*. Interestingly, this study found marked differences in cell infection between both genotypes of *T. copemani*. Irrespective of the cell line used, *T. copemani* G1 infection rates were very low, always below 7%. Although *T. copemani* G2 infection rates were slightly higher in some cell lines, this strain was highly infective to *VERO* cells, with 70% of cells infected at 48 hours post-infection. These results are consistent with previous findings (Chapters 2 and 3) (Botero *et al*. 2013), which showed that only *T. copemani* G2 was present in multiple marsupial host tissues, while *T. copemani* G1 was only found in blood. Taking this into account, it seems that the life cycle of both *T. copemani* G1 and G2 may differ considerably, therefore raising the question as to whether they are two different species. However, this requires further exploration.

T. cruzi as well as *T. copemani* G2 exhibited significant differences in infectivity between cell lines. *T. copemani* G2's rate of infection in *VERO* cells was double that the

rate of infection produced by *T. cruzi*. However, in *L6* and in both human derived cell lines, *T. cruzi* showed a higher level of cell invasion. It seems that the intensity of the infection depends on the cell line, or in other words, some cell lines are more susceptible to *Trypanosoma* infections. *In vitro* studies looking at the host cell interaction during infections with *T. cruzi* have demonstrated similar results (Yoshida et al, 2008). Furthermore, Andrade et al. (2010) suggested that the differences in infectivity and intracellular multiplication rates of *T. cruzi* in different cell lines are key factors influencing its tissue distribution in the vertebrate host. *T. cruzi* JG strain was shown to infect and replicate efficiently inside BALB/c cardiomyocytes *in vitro* (Andrade *et al*. 2010), and also exhibited a preferential development in cardiac tissue of experimentally infected BALB/c mice (Andrade *et al*. 1999). Interestingly, results that showed the kidney of naturally infected marsupials to be frequently infected with *T. copemani* G2 (Chapter 2 and 3) (Botero *et al*. 2013), are consistent with results of the present study in which the kidney epithelial derived *VERO* cell line was frequently invaded by *T. copemani* G2, thus supporting Andrade's hypothesis.

T. cruzi, which infects several mammals species and causes pathology in humans, dogs, and in experimentally infected mice and rats, has also been shown to produce an active infection in different cell lines isolated from humans, dogs, mice and rats *in vitro* (Barr *et al*. 1996, Andrade *et al*. 2010, Vargas-Zambrano *et al*. 2013). Moreover, a correspondence between *in vitro* studies and what is seen in natural infections has also been demonstrated in mosquitoes and ticks-borne viruses. It was shown that tick-derivated cell lines isolated in culture are susceptible to infection with tick-borne viruses and not to mosquito-borne viruses, while mosquito-derivated cell lines isolated in culture are susceptible to infection with mosquito-borne viruses and not to tick-borne

viruses (Lawrie *et al*. 2004). Interestingly, both *HCT8* and *THP1* (human derived) and *L6* (rat derived) cell lines exhibited a markedly lower infection with *T. copemani*, suggesting that there might be a low susceptibility of these hosts to both strains of the parasite. Smith *et al* (2008) examined Australian native wild rats for the presence of trypanosomes by PCR and sequencing of a fragment of the 18S rDNA gene using generic primers that recognise many species of trypanosomes, including *T. copemani*. Results showed some wild rats were infected with *T. lewisi,* but not with *T. copemani* (Smith *et al*. 2008). In a similar manner, the high infection rate found in the monkey derived *VERO* cells, might suggest an increased susceptibility of this host to *T. copemani* G2 infections. However, *T. copemani* has never been reported to naturally infect any other vertebrate host except marsupials. It has been demonstrated that culture-derived metacyclic forms of *T. cruzi* maintained for long periods of time (several years) in axenic culture have reduced capacity to invade cells and/or to replicate intracellularly, due to an alteration in the expression of proteins in the infective stage of the parasite (Contreras *et al*. 1994, 1998). However, the *T. copemani* strains used in this study have been maintained in culture for only one year.

The intracellular mechanism of replication in host tissues that some trypanosomes use evidently plays an important role in the pathogenesis of the parasite. Several studies have demonstrated that the presence of DNA and/or intact intracellular forms of *T. cruzi* in tissues is often accompanied by a strong inflammatory process triggered by the presence of the parasite in vital tissues such as heart, which ends in tissue damage and commonly in the death of the host (Tanowitz *et al*. 2009, Gutierrez *et al*. 2011, Corral *et al*. 2013) . The fact that *T. copemani* G2 was highly infective to one cell line proves the capacity of this parasite to infect tissue cells in the marsupial host and raises questions

about the potential virulence and pathogenicity of this parasite and its possible association with the rapid and substantial decline of the woylie (Chapter 3). Although a small number of *Trypanosoma* species have been shown to infect and multiply within host cells *in vitro*, including *T. dionsii*, *T. erneyi*, and *T. livingstonei* from bats, their potential pathogenicity in these animals has not been assessed (Oliveira *et al*. 2009, Lima *et al*. 2012, Lima *et al.* 2013).

Although some intracellular amastigotes were seen in cells infected with *T. copemani* G2, it was not comparable with that produced by *T. cruzi*. All cell lines infected with *T. cruzi* exhibited large numbers of intracellular amastigotes, indicating that cells were probably invaded more effectively and/or were more susceptible to intracellular replication by *T. cruzi*. Despite the rate of infection of *T. copemani* G2 in non-phagocytic *VERO* cells being high, no sign of active *Trypanosoma* multiplication was seen inside cells. This lack of intracellular replication of *T. copemani* G2 might be due to a number of different reasons. Firstly, it could be due to the inability of *T. copemani* to replicate inside cells. However, the findings of numerous structures suggestive of intracellular amastigotes of *T. copemani* G2 in woylie tissues (Chapter 3) (Botero *et al*. 2013) suggests that this parasite might be able to replicate inside tissue cells. Secondly, it has also been shown that the host genetic background plays an important role in susceptibility or resistance to infections with *T. cruzi* (Andrade *et al*. 2002), and therefore the origin of the cell line cannot be ruled out because it could determine the success or failure of the *in vitro* infections. It was shown previously that the intracellular multiplication efficiency of different strains of *T. cruzi* differs among cell lines (Andrade *et al*. 2010). Finally, there is the possibility that the nutritional conditions provided by the medium were sub-optimal to support the intracellular division of

T. copemani. Unfortunately, *T. copemani* is highly pleomorphic in culture and finding the optimal conditions (i.e. - nutrients in media, temperature, etc) to maintain all the different stages of the parasite in culture, including its infective stage is difficult to achieve, and therefore the conditions used may not have been the most appropriate to support the intracellular development of this parasite.

The similarity in the biological behaviour of *T. copemani* and *T. cruzi* suggests that these parasites might use similar strategies to complete their life cycle in the vertebrate host. However, the use of a better *in vitro* model, possibly a marsupial derived cell line, as well as complementary *in vivo* studies are required to fully understand the life cycle and virulence of *T. copemani* in Australian wildlife.

5.6 References

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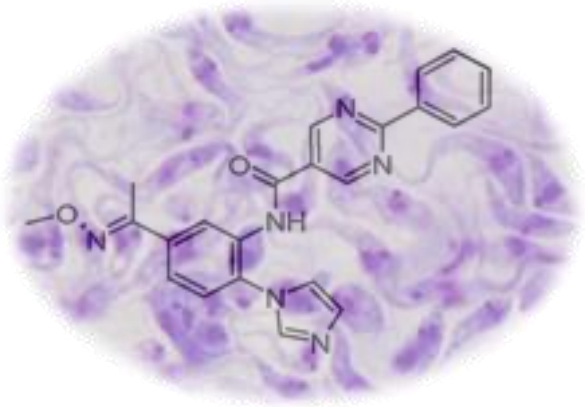
Chapter 6

In vitro drug susceptibility of two strains of Trypanosoma copemani: a comparison with the pathogenic Trypanosoma cruzi



6.1 Abstract

Trypanosomes are blood protozoan parasites that are capable of producing illness in the vertebrate host. Within Australia, several *Trypanosoma* species have been described infecting wildlife. However, only *Trypanosoma copemani* G2 has been suggested to produce harmful effects on the health of marsupials and has recently been associated with the dramatic decline of the woylie (*Bettongia penicillata*). The impact that some trypanosomes have on the health of the vertebrate host has led to the development of numerous drug compounds that could inhibit the growth or kill the parasite. This study investigated and compared the *in vitro* susceptibility of both *T. copemani* G1 and G2, and *T. cruzi* to drugs currently used against pathogenic trypanosomatids (benznidazole, posaconazole, miltefosine and melarsoprol) and to four new compounds (two pyridine and two fenarimols derivatives) developed primarily against *T. cruzi*. The *in vitro* cytotoxicity of all drugs against *L6* cells was also assessed. Results showed that both strains of *T. copemani* were more susceptible to all drugs and compounds than *T. cruzi*, with all IC50 values in the low and sub-μM range for both species. Melarsoprol and miltefosine exhibited the highest drug activity against both *T. copemani* and *T. cruzi*, but they also showed the highest toxicity in *L6* cells. Interestingly, fenarimol and pyridine compounds were more active against *T. cruzi* and *T. copemani* than the reference drugs benznidazole and posaconazole. *T. copemani* G1 and G2 exhibited differences in susceptibility to all drugs demonstrating once again considerable differences in their biological behaviour.

6.2 Background

Trypanosoma comprises a large number of species and subspecies that are capable of producing detrimental effects on the host. *T. cruzi* for example, is an intracellular protozoan that causes Chagas' disease in humans and is an important contributor to heart disease in Latin America (Kirchhoff. 1996). This parasite is able to infect different marsupial species in America and has been shown to produce inflammatory lesions in tissues similar to those seen in human infections (Barr *et al*. 1991, Carreira *et al*. 1996). Trypanosomes from the "*T. brucei* complex*"* are pathogenic trypanosomes from Africa that affect humans causing sleeping sickness, and animals causing nagana. Common signs of the infection in humans are swollen lymph nodes, fever, anaemia, oedema, neurological involvement, and rapid weight loss. Other trypanosomes considered non-pathogenic, may cause harm when they find a new or naïve vertebrate host. Within Australia, the accidental introduction of the exotic *T. lewisi* to Christmas Island caused a collapse in the population of the endemic rat *Rattus macleari* to the point of complete extinction (Pickering *et al*. 1996, Wyatt *et al*. 2008). More recently, a native Australian trypanosome, *T. copemani* G2, was associated with the rapid and substantial population decline (90% within 10 years) of the critically endangered marsupial, the woylie (*Bettongia penicillata*) (Wayne *et al*. 2013). This parasite has been shown to colonise several tissues in the marsupial host, with evidence of cell invasion, and production of extensive inflammatory cell infiltrates and tissue damage (Chapter 3) (Botero *et al*. 2013), thus demonstrating a pathogenic potential previously not associated with trypanosomes of wildlife from Australia.

The impact that pathogenic trypanosomes have on the health of the vertebrate host has led to the development of numerous drug compounds that could inhibit or kill the

parasite. Drugs currently used to treat Chagas disease and that have been the focus of many *in vitro* studies include benznidazole, and posaconazole. Benznidazole (N-benzyl-2-nitro-1-imidazole-acetamide) is currently used in the treatment of *T. cruzi* infections. Despite this drug not being completely effective, especially in the chronic stage of the disease ((WHO. 2007, Soeiro *et al*. 2009, Batista *et al*. 2011), it is one of the main drug therapies available to treat the disease. Posaconazole is an ergosterol biosynthesis inhibitor that has also shown potent *in vitro* and *in vivo* activity against *T. cruzi* (de Figueiredo Diniz *et al*. 2013). However, a recent clinical trial of posaconazole in 78 adults with chronic *T. cruzi* infections showed a very poor efficacy of this drug, and greater percentage of treatment failure when compared with benznidazole treated patients (Molina *et al*. 2014).

On the other hand, drugs currently used to treat human but also livestock trypanosomiasis caused by the *T. brucei* complex include melarsoprol and miltefosine. Melarsoprol is an arsenical drug that has been used against late-stage infections with *T. brucei* subspecies (Denise *et al*. 2001), and miltefosine is an alkylphosphocholine that was the first and still the only oral drug that can be used to treat visceral and cutaneous Leishmaniasis, a disease caused by protozoan parasites of the *Leishmania* genus (Dorlo *et al*. 2012). Although, all these drugs are the main treatment used to combat trypanosomatids infections, they are less than ideal due to toxicity and adverse side effects (Castro *et al*. 2006, Hasslocher-Moreno *et al*. 2012, Pinazo *et al*. 2013).

CYP51 is an enzyme of critical importance to organisms that require sterol biosynthesis for membrane function. Inhibition of *T. cruzi* CYP51 has been shown to affect sterol composition and damages parasite ultrastructure leading to the death of the parasite

(Lepesheva *et al*. 2011). Attempts to develop new compounds with potent activity against trypanosomes and low toxicity in mammalian cells has led to the discovery of different ergosterol biosynthesis inhibitor compounds with demonstrated *in vitro* and *in vivo* activity against all *T. brucei* subspecies and *T. cruzi*. Most of these compounds have exhibited good activity against trypanosomes, low toxicity in mammalian cells and good pharmacokinetic properties in the vertebrate host suggesting suitable drug-like properties for preclinical evaluation (Keenan *et al*. 2012, Keenan *et al*. 2013, Hargrove *et al*. 2013).

Considering not only the potential pathogenicity of *T. copemani* G2 in the woylie, but also that this parasite has been found infecting other critically endangered and vulnerable Australian marsupials such as quokkas (*Setonix brachyurus*), chuditches (*Dasyurus geoffroii*), and southern brown bandicoots (*Isoodon obesulus*) (Chapter 3) (Botero *et al*. 2013) there is a need to evaluate the susceptibility of *T. copemani* to drugs that can be used to protect wildlife health and conservation. Evaluating and comparing the drug susceptibility of both *T. copemani* G1 and G2 might also provide new evidence to suggest their status as separate species. Therefore, this chapter aims to investigate and compare the *in vitro* susceptibility of *T. copemani* G1 and G2, and *T. cruzi* to reference drugs and compounds currently used against pathogenic trypanosomatids.

6.3 Materials and methods

6.3.1 Parasites

Trypanosoma copemani strains G1 (Genotype 1) and G2 (Genotype 2) isolated from the blood of woylies (Chapter 4) (Botero *et al*. 2013), and *T. cruzi* 10R26 strain were used

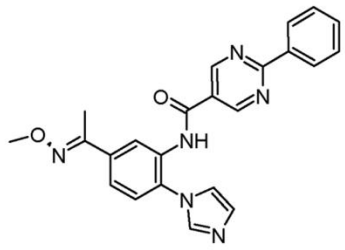
and maintained as epimastigotes by successive passages every 3 days at 28°C in RPMI medium containing 10% of FCS, 5mg/ml of penicillin-streptomycin and 2.5mg/L of haemin.

6.3.2 Cell culture

For drug toxicity assays, *L6* cells (skeletal myoblast cells) purchased from the American Type Culture Collection were used. Cells were grown in RPMI medium supplemented with 10% of foetal calf serum (FCS) at 37°C and 5% of CO_2.

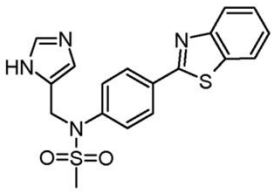
6.3.3 Test compounds

Miltefosine and melarsoprol were kindly provided by Dr Vanessa Yardley (London School of Hygiene and Tropical Medicine, UK). Benznidazole tablets (Rochagan - 100 mg) were purchased from Roche (Rio de Janeiro, Brazil). Posaconazole was purchased as an oral suspension (Noxafil Schering Corporation, 40 mg/mL) and isolated from the suspension by dilution with water and centrifugation, followed by extraction and recrystallization from hot i-propyl alcohol (Keenan *et al*. 2012). Four CYP51 inhibitor compounds, two pyridine derivatives (EPL-BS967 or PDB1 and EPL-BS1246 or PDB2) and two non-azole antifungal fenarimoles (EPL-BS1937 or FN1 and EPL-BS2391 or FN2) were developed, synthetised and kindly provided by Epichem Pty Ltd. Their molecular structures are shown in Figure 1. Drug compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at 4 ºC. Immediately before use, drugs were pre-diluted in RPMI media to the desired concentration. The final DMSO concentration did not exceed 1% (v/v) and had no effect by itself on the proliferation of the parasites.



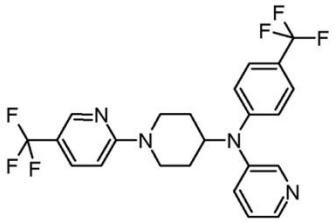
EPL-BS1937 **(FN1)**

N-(2-(1*H*-imidazol-1-yl)-5-[(1E)-*N*-methoxyethanimidoyl]phenyl)-2-phenylpyrimidine-5-carboxamide



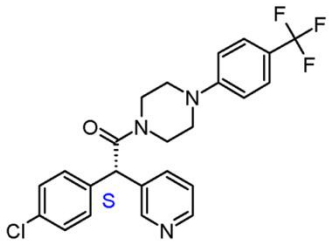
EPL-BS2391 **(FN2)**

N-[4-(1,3-benzothiazol-2-yl)phenyl]-*N*-(1*H*-imidazol-5-ylmethyl)methanesulfonamide



EPL-BS967 **(PDB1)**

N-[4-(trifluoromethyl)phenyl]-N-(1-[5-(trifluoromethyl)pyridin-2-yl]piperidin-4-yl)pyridin-3-amine



EPL-BS1246 **(PDB2)**

(S)-2-(4-Chlorophenyl)-1-{4-[4-(trifluoromethyl)phenyl]piperazin-1-yl}-2-(pyridin-3-yl)ethanone

Figure 1. Molecular structure of the two fenarimols and two pyridine compounds.

6.3.4 In vitro compound activity against trypanosomes

Epimastigotes of *T. copemani* G1 and G2 and *T. cruzi* 10R26 strains in the log phase of growth were diluted in RPMI media to $1x10^6$ parasites/ml. 100 µl of parasite suspension ($1x10^5$ parasites/well) was seeded into 96-well flat-bottom plates (Corning, Corning, N.Y.), and then incubated at 28 ºC in a seven-fold dilution series covering a range from 1 µM to 0.004 µM for melarsoprol and ranging from 10 µM to 0.013 µM for the rest of the drugs. Each drug concentration was evaluated in triplicate. Control wells with only compounds and with only parasites (without compounds) were included. After 48 hours of compounds exposure, 15 µl of AlamarBlue® (Resazurin - AbD Serotec) was added to each plate allowing for a colour change through metabolic oxidation-reduction by

viable trypanosomes. Plates were incubated for an additional 24 hours. After this time, absorbance was quantified using a Dynex microplate reader at an excitation wavelength of 570 nm and emission wavelength of 590 nm. The percentage of inhibition was calculated by the following equation:

$$\textbf{\% Inhibition} = \frac{100 - [(\text{Trypanosomes with compound} - \text{compound only})}{(\text{Trypanosomes only} - \text{media only})] \times 100}$$

For each compound, percentage of inhibition values were used to generate dose-response curves by average of triplicate data points. The concentration (μM) of the drug necessary to inhibit 50% of cell proliferation of that observed in control cultures (parasites grown in the absence of test compound) was calculated (IC50). Graphs were created and analysed using the statistical software program Prism (GraphPad Software Inc., San Diego, Cali). The statistical significance of results was estimated by 2way ANOVA. Each experiment was performed on three independent occasions.

6.3.5 In vitro compound toxicity in L6 cells

An evaluation of mammalian cell cytotoxicity was carried out in parallel. 100 μL of RPMI 1640 medium supplemented with 10% fetal bovine serum and containing 5 x 10^3 *L6* cells were seeded into 96-well plates. Plates were incubated overnight and drugged with seven 3-fold-dilutions covering a range from 10 μM to 0.013 μM for melarsoprol and miltefosine, and 100 μM to 0.13 μM for the rest of the drugs. Control wells with only compounds and with only cells were included. After 72 hours of incubation with the drugs, plates were inspected under an inverted microscope to assure growth of cells in the control wells (no drugged) and sterile conditions. 15 μL of AlamarBlue® was then added to wells and the plates incubated for another 2 hours. Absorbance was

quantified using a Dynex microplate reader at an excitation wavelength of 570 nm and emission wavelength of 590 nm. Podophyllotoxin was used as a reference drug for toxicity. The therapeutic index (TI) of all drugs was calculated as TD50/ED50, where TD50 is the dose of drug that causes a toxic response in 50% of the *L6* cells (IC50 value for cytotoxicity) and ED50 is the dose of drug that is active in 50% of trypanosomes (IC50 value for anti-trypanosomal activity), as shown in Figure 2. The statistical significance of results was estimated by 2way ANOVA. Each experiment was performed on three independent occasions.

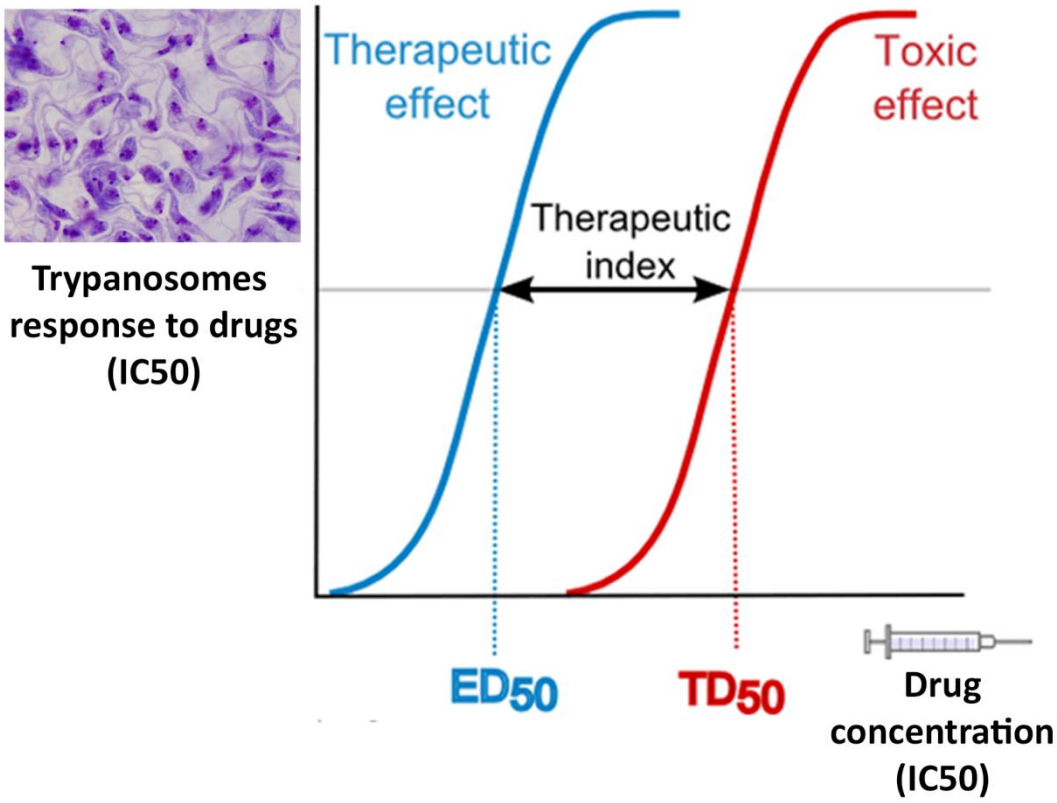


Figure 2. How the therapeutic index was determined.
Modified from http://pharmacologycorner.com/therapeutic-index

6.4 Results

6.4.1 In vitro compound efficacy of reference drugs

The reduction of resazurin, converted from blue to a bright-red colour by metabolically active trypanosomes/cells, was used as an indicator of cell/trypanosome viability and therefore as a measure of drug activity/toxicity (Figure 3).

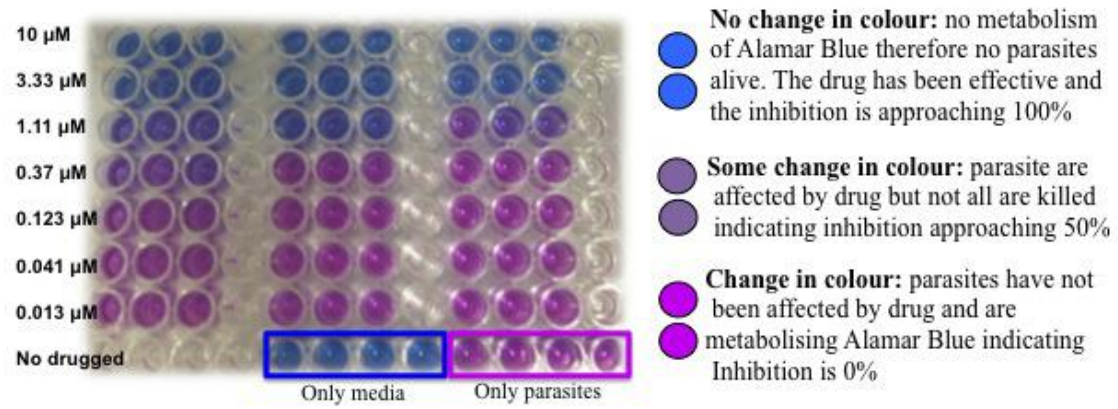


Figure 3. Percentage of inhibition of posaconazole against *T. copemani* G1. This figure illustrates the percentage of inhibition (change of colour) in tissue culture plate-wells (three replicates with three data points each) containing *T. copemani* parasites drugged with different concentrations of posaconazole ranging from 10 µM to 0.013 µM. Clear wells contain only medium.

All reference drugs exhibited potent *in vitro* activity against all trypanosomes. However, both strains of *T. copemani* were more susceptible to all drugs than *T. cruzi*. Benznidazole was approximately eight times more active against *T. copemani* G1 (IC50 1.053 µM) and G2 (IC50 0.713 µM) than against *T. cruzi* (IC50 8.537 µM) (Figure 4).

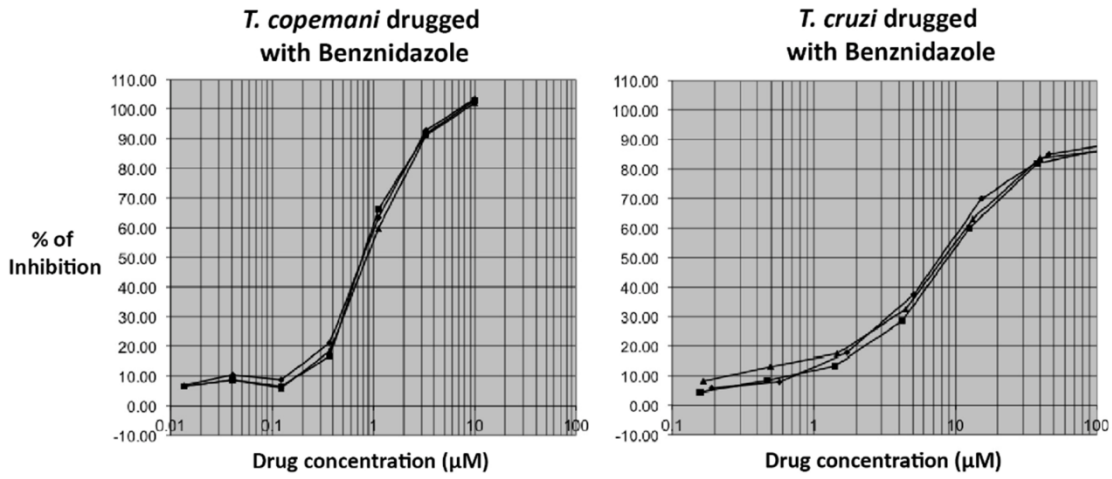


Figure 4. Sigmoidal dose-response curves of *T. copemani* G2 and *T. cruzi* drugged with benznidazole. X-axis: percentage of inhibition. Y-axis: drug concentration.

Posaconazole exhibited a similar activity against *T. cruzi* and *T. copemani* G2, both with IC50 of 5.429 µM and 6.147 µM respectively. This drug was more active against *T. copemani* G1, which exhibited an IC50 of 1.254 µM. Melarsoprol and miltefosine were the most active drugs against all parasites tested. However, melarsoprol was much more active with IC50s in the sub-µM range. Significant differences in drug susceptibility between *T. copemani* G1 and G2 ($p<0.0001$) were found. *T. copemani* G2 was more susceptible to benznidazole and melarsoprol. In contrast, *T. copemani* G1 was more susceptible to melarsoprol and miltefosine (Table 1, Figure 5).

Table 1. Inhibitory concentration 50 (IC50) of all reference drugs against *T. copemani* G1 and G2, and *T. cruzi* and toxicity against *L6* cells. Values are in µM. SD: standard deviation.

Compounds	*T. copemani* G1 (IC50±SD)	*T. copemani* G2 (IC50±SD)	*T. cruzi* (IC50±SD)	Toxicity on *L6* cells (IC50±SD)
Benznidazole	1.053 ± 0.183 (>94.9)	0.713 ± 0.186 (>140.2)	8.537 ± 0.306 (>11.7)	>100 µM
Posaconazole	1.254 ± 0.418 (>79.7)	6.147 ± 0.154 (>12.3)	5.429 ± 0.151 (>18.4)	>100 µM
Melarsoprol	0.007 ± 0.001 (8.8)	0.005 ± 0.0006 (12.1)	0.010 ± 0.001 (6.2)	0.062 µM
Miltefosine	0.095 ± 0.007 (2.4)	0.745 ± 0.034 (0.31)	2.109 ± 0.112 (0.1)	0.231 µM
Podophyllotoxin[a]	-	-	-	0.01 µM

[a]: reference drug for toxicity
[0] Therapeutic indices are given in parenthesis

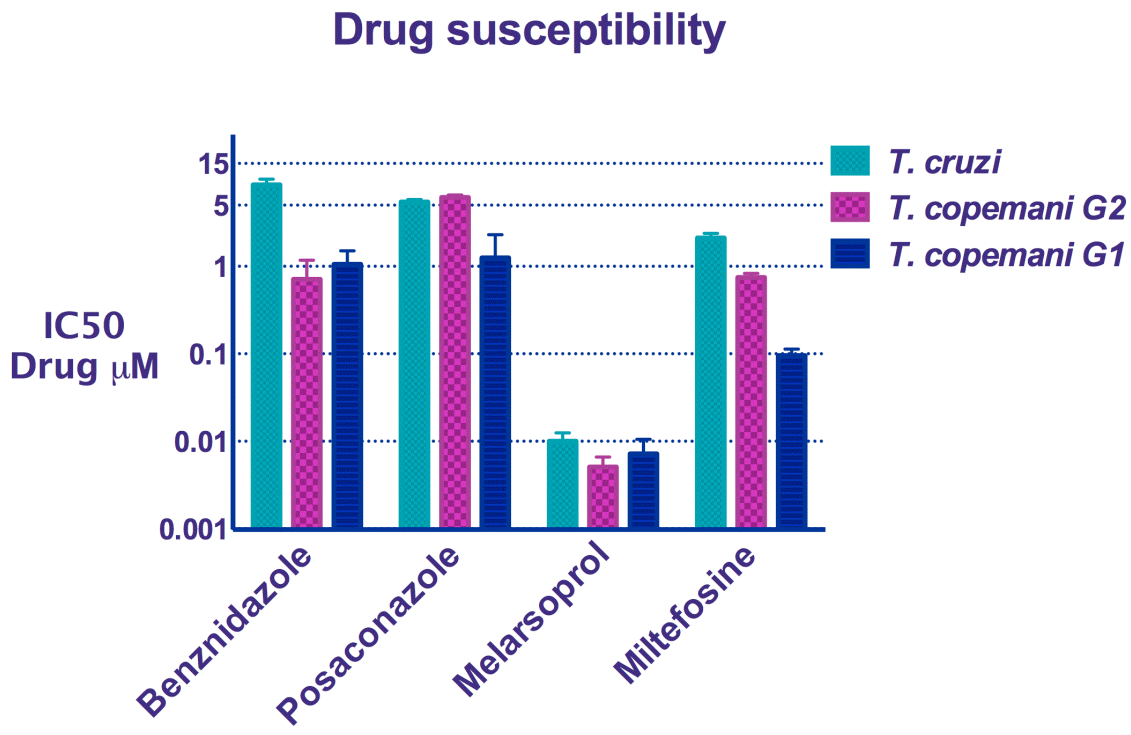


Figure 5. Drug susceptibility of *T. copemani* G1, G2, and *T. cruzi* against reference drugs. X-axis: IC50. Y-axis: drugs. Bars: standard deviation.

6.4.2 In vitro compound efficacy of new compounds

Both fenarimols and both pyridine derivatives exhibited potent *in vitro* activity against all trypanosomes in the low and sub-μM range. However, they were more active against both strains of *T. copemani*, with the exception of PDB1 that was less active against *T. copemani* G2 than *T. cruzi* (Table 2). All four compounds exhibited similar activity against *T. cruzi*, with IC50 values ranging from 4.5 μM to 6.1 μM. FN2 was the compound that presented the highest activity against both *T. copemani* G1 and G2, with IC50 of 1.122 μM for G1 and 0.969 μM for G2. There was a significant difference in susceptibility of both *T. copemani* strains to all compounds ($p<0.0001$), with *T. copemani* G2 more susceptible to fenarimol compounds and *T. copemani* G1 more susceptible to pyridine derivative compounds (Table 2, Figure 6).

Table 2. Inhibitory concentration 50 (IC50) of fenarimols and pyridine derivatives against *T. copemani* G1 and G2, and *T. cruzi* and toxicity against *L6* cells. Values are in μM. SD: standard deviation. FN: fenarimol derivatives. PDB: pyridine derivatives.

Compounds	***T. copemani* G1 (IC50±SD)**	***T. copemani* G2 (IC50±SD)**	***T. cruzi* (IC50±SD)**	**Toxicity on *L6* cells (IC50±SD)**
FN1	3.316 ± 0.1021 (>30.1)	2.395 ± 0.302 (>41.7)	6.112 ± 0.0655 (>16.4)	>100 μM
FN2	1.122 ± 0.3971 (53.1)	0.969 ± 0.188 (61.4)	5.979 ± 0.2281 (10)	59.52 μM
PDB1	2.675 ± 0.7263 (>37.4)	7.178 ± 0.713 (>14)	5.261 ± 0.6828 (>19)	>100 μM
PDB2	1.51 ± 0.2736 (33.1)	3.343 ± 0.197 (15)	4.533 ± 0.3151 (11)	50.06 μM
Podophyllotixin[a]	-	-	-	0.01 μM

[a]: reference drug for toxicity
[0] Therapeutic indices are given in parenthesis

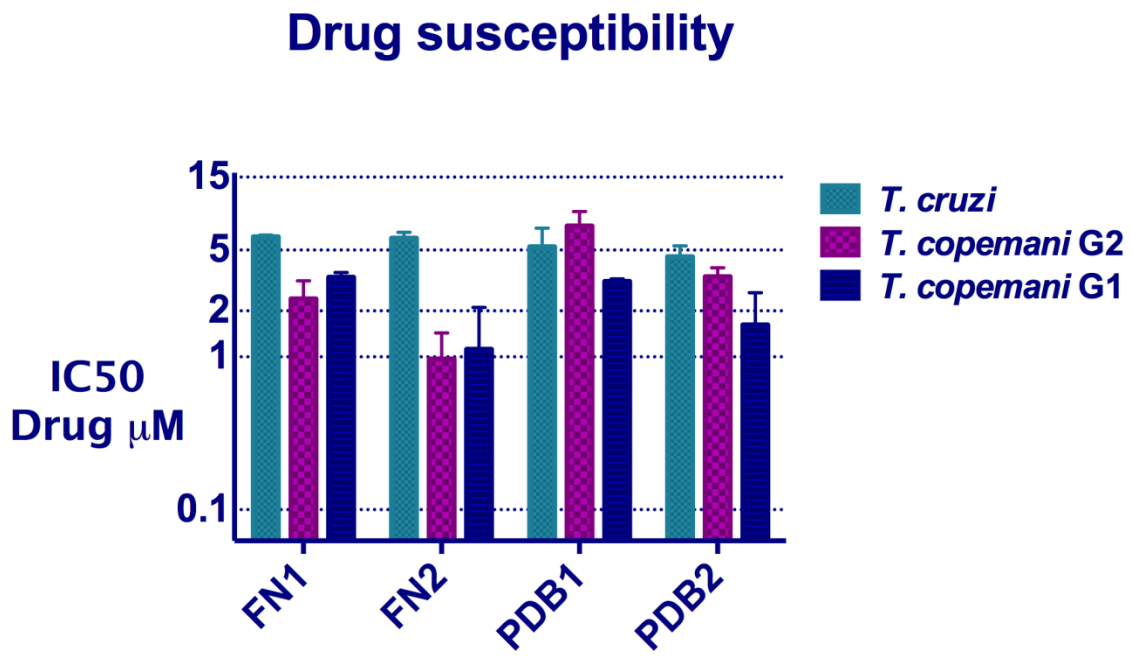


Figure 6. Drug susceptibility of *T. copemani* G1, G2, and *T. cruzi* against the new fenarimol and pyridine derivatives. X-axis: IC50. Y-axis: drugs. Bars: standard deviation.

6.4.3 In vitro drug toxicity in L6 cells

Cells were exposed to various concentrations of each drug, and viable cell number was determined by the AlamarBlue® metabolic assay. The therapeutic indices (TI = IC50 value for cytotoxicity divided by IC50 value for antiprotozoal activity) of all compounds were calculated and given for each parasite (Tables 1 and 2). The higher the TI, the better the drug is due to the very small dose of the drug that is needed to be effective.

The highest cytotoxicity for *L6* cells was exerted by melarsoprol (IC50, 0.062 μM) and miltefosine (IC50, 0.231 μM), which, interestingly, had the highest activity against all trypanosomes as well (Table 1). Furthermore, TI of both drugs was in general

significantly low (Melarsoprol TI <12.1 and Miltefosine TI <2.4) suggesting the effect of the drug was in part due to cytotoxicity instead of only to anti-trypanosomal activity (Table 1).

FN2 and PDB2 compounds exhibited IC50s of 59.52 μM and 50.06 μM respectively in *L6* cells, followed by benznidazole, posaconazole, FN1 and PDB1, which exhibited IC50s bigger than 100 μM. However, benznidazole and FN1 presented better therapeutic indices against both *T. copemani* strains, and PDB1 exhibited a better therapeutic index against *T. cruzi* than benznidazole and posaconazole (Tables 1 and 2).

6.5 Discussion

The effect of different drugs and new compounds on the growth of two strains of *T. copemani* and one strain of *T. cruzi* was investigated and compared using the AlamarBlue® assay. The AlamarBlue® assay is a simple, sensitive and reproducible method to measure the viability of different cell lines (Ansar Ahmed *et al*. 1994). It has been extensively used to determine the *in vitro* activity/toxicity of different drugs against different trypanosomatids such as *T. cruzi*, *T. brucei* and *Leishmania* spp. (Rolón *et al*. 2006, Morais-Teixeira *et al*. 2011). A previous study found AlamarBlue® was a good method to quantify the activity of different compounds against *T. brucei gambiense* and *T. b. rhodesiense in vitro* and demonstrated that results were comparable to those obtained with other fluorochrome dyes (Räz *et al*. 1997). Furthermore, it has been shown to be slightly superior in sensitivity to the MTT cell proliferation assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which has been extensively used in high throughput screenings (Hamid *et al*. 2004, Ho *et al*. 2012). The

results of the present study showed IC50 values for benznidazole and miltefosine similar to IC50 values reported in previous studies for *T. cruzi,* confirming the reliability and reproducibility of this assay (Santa-Rita *et al*. 2000, Lira *et al*. 2001, Saraiva *et al*. 2002, Luna *et al*. 2009).

This is the first study looking at the *in vitro* susceptibility of Australian trypanosomes to different drugs and new compounds developed against different trypanosomatids. All reference drugs benznidazole, posaconazole, melarsoprol and miltefosine, displayed promising trypanocidal activity against both strains of *T. copemani* isolated from the critically endangered marsupial the woylie and against *T. cruzi*, showing a broad anti-trypanosomal spectrum. Previous studies have also demonstrated that some of these reference drugs present a broad-spectrum of activity. Miltefosine for example, originally developed as an anticancer agent and now used for treatment of both visceral and cutaneous leishmaniasis, has also been shown to be active *in vitro* against *T. cruzi*, with IC50 ranging from 1 μM to 3.5 μM (Santa-Rita *et al*. 2000, Lira *et al*. 2001, Saraiva *et al*. 2002). Melarsoprol, an organoarsenic compound that is mainly used against late-stage sleeping sickness caused by *T. brucei* subspecies (Schweingruber. 2004), has also been shown to be active *in vitro* and *in vivo* against *T. lewisi*, a trypanosome that infect rats (Howie *et al*. 2006, Verma *et al*. 2011, Dethoua *et al*. 2013)

Miltefosine was highly active against *T. cruzi* and *T. copemani* G1 and G2, with IC50 of 0.095 μM, 0.745 μM and 2.1 μM respectively. However, it has been shown to present a significantly lower activity *in vitro* and *in vivo* against *T. brucei* subspecies with 18-fold and 43-fold greater IC50 values of 35.5 μM for *T. brucei brucei* and 47.0 μM for *T. brucei rhodesiense* in *in vitro* experiments (Croft *et al*. 1996), and 76 μM for

T. brucei gambiense and 88 μM for *T. brucei rhodesiense* in experimentally infected mice (Konstantinov *et al*. 1997). The significant differences in miltefosine activity between species is not surprising if we take into account the fact that antiparasitic drugs are usually developed to target and/or inhibit intracellular signaling pathways that are crucial in cell replication and survival, and those pathways may differ between species. Hence, the significant similarities in the activity of miltefosine against both *T. cruzi* and *T. copemani* may be due to intrinsic similarities between them in the target site of the drug. The mechanism of action of miltefosine in *T. cruzi* seems to be related to the inhibition of *the novo* phosphatidylcholine biosynthesis and phospholipid signaling pathways (Croft *et al*. 1996, Malaquias *et al*. 1999). However, the underlying mechanism of action of miltefosine against Australian *Trypanosoma* species is unknown, and although it could be the same pathway used in *T. cruzi*, it needs to be further investigated.

Surprisingly, benznidazole and posaconazole, drugs currently used to combat *T. cruzi* infections, presented lower activity against this parasite than the drugs miltefosine and melarsoprol. Similar studies evaluating the susceptibility of different strains of *T. cruzi* to miltefosine found this drug presented a greater activity against each strain than the reference drug benznidazole with IC50s ranging between 0.9 μM to 3.0 μM for miltefosine and 9.0 μM to 27 μM for benznidazole (Saraiva *et al*. 2002, Luna *et al*. 2009). However, it cannot be ignored that miltefosine and melarsoprol exhibited the highest toxicity to the mammalian cell line used and the lowest therapeutic indices. This suggests that the greater activity of both drugs against *T. cruzi* and *T. copemani* may in part be due to drug cytotoxicity and not entirely due to their trypanocidal activity. This is not the first study showing cytotoxicity of melarsoprol and miltefosine. Melarsoprol

has been shown to induce programmed cell death or apoptosis in leukemic and plasma cell lines *in vitro* (König *et al*. 1997, Rousselot *et al*. 1999) as well as miltefosine in numerous tumour cell lines (Engelmann *et al*. 1995, Henke *et al*. 1998, Rybczynska *et al*. 2001).

All fenarimol and pyrimidine derivatives exhibited potent activity against *T. cruzi* and *T. copemani* epimastigotes. Moreover, both fenarimol and pyrimidine compounds presented a better activity against *T. cruzi* than the *T. cruzi* reference drug benznidazole. These results are consistent with those obtained by Keenan (Keenan *et al*. 2013), who showed that both FN1 and FN2 exhibited a curative activity in mice infected with *T. cruzi* Tulahuen strain and a significant activity *in vitro* against *T. cruzi* amastigotes, as well as low toxicity in *L6* cells. However, the *T. cruzi* IC50s of both fenarimol compounds obtained in the present study are generally higher than those previously reported (Keenan *et al*. 2013). These discrepancies derive from the use of different *T. cruzi* strains but most probably may derive from the use of different trypanosome life cycle stages in both studies. Several studies have revealed that some drugs or compounds, including benznidazole are more active against intracellular amastigotes than against axenically grown epimastigotes of *T. cruzi* (Freire-de-Lima *et al*. 2008, Luna *et al*. 2009). It has also been shown with *Leishmania*, that promastigotes tended to be less sensitive than intracellular stages to different drugs (Vermeersch *et al*. 2009). Those results might be attributed to cell-mediated antiparasitic mechanisms of the drugs.

T. copemani G1 and G2, although grouped within the same clade in a phylogeny, exhibited genetic differences with both 18SrDNA and g*GAPDH* loci (Chapter 2) (Botero *et al*. 2013). Interestingly, the present study showed that both strains of

T. copemani exhibited significant differences in susceptibility to the different drugs used, supporting previous hypotheses suggesting that genetic variation within species could determine the degree of susceptibility to drugs (Campos *et al*. 2011, Plourde *et al*. 2012). Previous studies have shown an association between *T. cruzi* genetic diversity and their susceptibility to different drugs. Toledo (de Ornelas Toledo *et al*. 2003) reported a different response to the drugs benznidazole and itraconazole among genetically different *T. cruzi* group I and group II strains. Moreover, the observation of differences in susceptibility to benznidazole among several *T. cruzi* strains isolated from humans, vectors and marsupials has also been reported (Toledo *et al*. 1997). Phylogenetic studies have shown a considerable intra-specific genetic variability within *T. copemani* isolates and the presence of co-infections with different *T. copemani* genotypes/strains in naturally infected animals (Botero *et al*. 2013). This variability and its possible association with the different phenotypic responses to drugs may complicate the scenario and may have important consequences on future attempts to combat *T. copemani* infections. However, it can not be ignored that *T. copemani* G1 and G2 have exhibited other phenotypic differences distinct to drug susceptibility, such as different growth kinetics in culture (Chapter 4) and different cell infection *in vitro* (Chapter 5), indicating once again that they might correspond to different *Trypanosoma* species.

Finally, the fact that benznidazole and FN2 had the highest therapeutic indices against *T. copemani* G1 and G2 (Benznidazole TI> 94.9 and TI>140.2; FN2 TI> 53.1 and TI>61.4) suggests these drugs could be used as potential therapeutics for ameliorating the clinical effects of infections with this parasite. Therefore, they may be of conservation value in managing the declines of naturally infected native marsupials in

the future.

6.6 References

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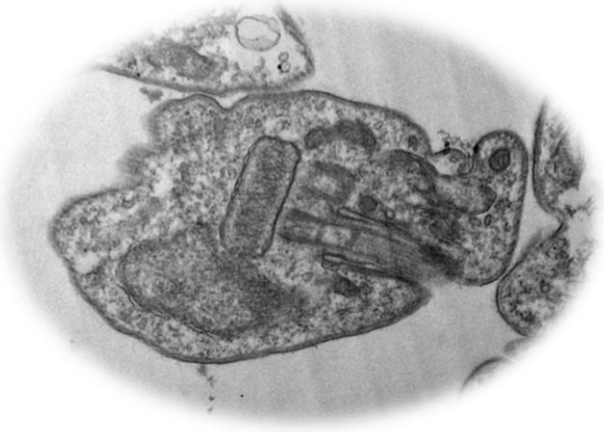
Chapter 7

Novel insights into the Kinetoplast DNA structure of the Australian Trypanosoma copemani



7.1 Abstract

The kinetoplast is an organelle that is present in all trypanosomatids. It contains a giant network of thousands of catenated circular DNAs (kDNA) with unique structure and function. KDNA consists of a few dozen maxicircles that encode mitochondrial gene products, and several thousand minicircles that encode guide RNAs for the editing of mitochondrial RNA transcripts. Minicircles have been extensively used in the development of sensitive and specific diagnostic molecular tools due to their abundance and heterogeneity in size and sequence between species. They contain a 12-nucleotide-sequence named "The Universal Minicircle Sequence" (UMS) that is conserved within most trypanosomatids. The number of UMS elements and their location in each minicircle differ between species. This chapter reports novel insights into the kinetoplast structure and kDNA organisation of *Trypanosoma copemani*, a parasite associated with the drastic decline of the Australian marsupial *Bettongia penicillata*. Transmission electron microscopy images showed classical disk-shaped kDNA network morphology, similar to that seen in late-emerging trypanosomatids such as *T. cruzi*, *T. brucei* and *C. fasciculata*. PCR, sequencing, and Western blot analysis showed the presence of the UMS elements in the minicircles of *T. copemani* and the existence of the UMS-Binding Protein (UMSBP), which is also present in all trypanosomatids and is involved in minicircles replication. Sequences obtained from the minicircles of *T. copemani* G1 and *T. copemani* G2 strains revealed significant similarities with the minicircles of *T. cruzi*.

7.2 Background

The kinetoplast is an organelle that contains the mitochondrial DNA (kDNA) of trypanosomatid protozoa. The kinetoplast DNA or kDNA consists of thousands of interlocked or catenated DNA circles in two forms, maxicircles and minicircles that form a complex network (Lukeš *et al*. 2002, Jensen *et al*. 2012). Maxicircles comprise only a small portion of the kDNA network, with only a few dozen identical copies. Their size range from about 20 to 40 Kb, according to the species, and their function is to encode mitochondrial gene products (Shlomai. 2004). In contrast to maxicircles, minicircles comprise a big portion of the kDNA network and are present in several thousand copies that differ in size and sequence according to the species (Ray. 1987). Minicircles encode guide RNAs (gRNAs) that contain the genetic information for editing of mitochondrial RNA transcripts (Lukeš *et al*. 2002).

A huge diversity in kDNA structure and conformation has been demonstrated within different kinetoplastids including free-living microorganisms and trypanosomatid protozoa. A classical disk-shaped kDNA network has been reported in *T. cruzi*, *T. brucei, L. tarentolae* and *C. fasciculata.* Minicircles within the network are catenated, and are released from the network through decatenation by DNA topoisomerase II enzymes prior to their replication (Shapiro *et al*. 1995). In contrast, the minicircles in species of the early and late branching family Bodonidae such as *Bodo caudatus* and others are not organised in a network. Instead, they are distributed in diverse forms across the mitochondrial matrix known as poly-kDNA, pan-kDNA, mega-kDNA, and pro-kDNA (Vickerman. 1990, Lukeš *et al*. 2002). Correlations between these diverse patterns of kDNA organization and genetic analysis based on nuclear rRNA genes have

contributed to a better understanding of the evolution of kDNA and have facilitated the establishment of phylogenetic relationships between kinetoplastids (Lukeš *et al*. 2002).

A common feature within minicircles is the presence of a 12-nucleotide sequence named "Universal Minicircle Sequence" (UMS) that is conserved in most trypanosomatids and is part of the minicircle replication origin (Ray. 1989). However, the number of the UMS elements, their location in each minicircle, and the size of each minicircle differ among the species (Ponzi *et al*. 1984, Sugisaki *et al*. 1987b, Degrave *et al*. 1988). The regions of the minicircles flanked by the different UMS elements are heterogeneous in sequence and have been used to investigate intraspecific variations within species (Telleria *et al*. 2006). Due to the minicircle abundance and heterogeneity in sequence, they have been used considerably in the development of sensitive and specific diagnostic molecular tools using PCR (Noyes *et al*. 1998, Botero *et al*. 2010, Ceccarelli *et al*. 2014). The UMS is the specific binding site for the UMS-Binding Protein (UMSBP), a protein involved in kDNA replication (Tzfati *et al*. 1995b). The UMSBP of *C. fasciculata* for example, has been extensively studied (Onn *et al*. 2006). It was shown that its UMSBP is a single-stranded sequence-specific DNA binding protein that binds the UMS (12-mer) and a hexameric sequence (Abu-Elneel *et al*. 1999) that are conserved at the replication origins of *C. fasciculata* kDNA minicircles, as well as in the minicircles of many trypanosomatid species (Ray. 1989). Recent studies using antibodies raised against *C. fasciculata* UMSBP have found the presence of *C. fasciculata* UMSBP orthologues in other trypanosomatids such as *T. cruzi* and *T. brucei* (Coelho *et al*. 2003, Milman *et al*. 2007). These results were not surprising considering that the replication origin sequences of minicircles in all trypanosomatids are highly conserved. The importance of the UMSBP in the survival of trypanosomatids was demonstrated using RNA interference (RNA*i*) experiments. It was shown that

knocking down UMSBPs by RNA*i* not only affects the initiation of minicircle replication, but also inhibits segregation of the daughter networks and blocks nuclear division (Milman *et al*. 2007), suggesting this protein as a potential drug target against pathogenic trypanosomes.

The kDNA has been of major interest not only because of its peculiar mitochondrial genome organisation, but also because its heterogeneity between species can be a clue to understanding the evolutionary history of trypanosomatids (Simpson *et al*. 2002). Although several species and genotypes of Australian trypanosomes have been described since the 50's (Mackerras. 1959, Noyes *et al*. 1998, Smith *et al*. 2008, Austen *et al*. 2009, Averis *et al*. 2009, McInnes *et al*. 2009, McInnes *et al*. 2011, Paparini *et al*. 2011, Thompson *et al*. 2013), there is a complete lack of knowledge about their kinetoplast and the organization of the DNA (kDNA) within this organelle. Therefore, the aims of this study were to investigate the ultrastructure and organization of the kDNA of the Australian *T. copemani,* including the organisation of the minicircles and the presence of the Universal Minicircle Sequence (UMS) element and the UMS-Binding Protein (UMSBP), which are present in all trypanosomatids studied.

7.3 Materials and methods

7.3.1 Parasites

Epimastigotes of *T. copemani* G1 and G2 were grown in 75 ml flasks containing Grace's medium with 10% of FCS and penicillin-streptomycin. *T. cruzi* epimastigotes were grown in 75 ml flasks containing RPMI 1640 media with 10% of FCS and

penicillin-streptomycin and *C. fasciculata* cultures were grown with agitation (150–200 rpm) in brain-heart infusion media (BHI- Difco) containing 10 mg/ml haemin. All strains were grown at 28° C and 5% of CO_2.

7.3.2 Transmission Electron microscopy

T. copemani G1 and G2 epimastigotes were fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS: cell culture medium (pH 7.2). All subsequent processing was performed in a PELCO Biowave microwave, where samples were post-fixed in 1% OsO_4 (osmium tetroxide) in PBS (Phosphate buffered saline) followed by progressive dehydration in ethanol/acetone, before being infiltrated and embedded in epoxy resin Procure-Araldite. 120nm-thick sections were cut with a diamond knife and mounted on copper grids. Digital images were collected from unstained sections at 120kV on a JEOL 2100 TEM fitted with a Gatan ORIUS1000 camera. The thickness and length of the kinetoplast were measured in trypanosome sections where the basal body of the flagellum was seen and where most of the DNA fibers within the kinetoplast were continuously distributed from side to side (indicating that the kinetoplast disk was cut parallel to its long axis and through its central region respectively).

7.3.3 Isolation of kinetoplast DNA networks

Ethidium bromide-cesium chloride stepwise gradients were used to isolate the kDNA of *T. copemani* and *C. fasciculata* (Saucier *et al*. 1981, Hajduk *et al*. 1984). $1x10^{10}$ *T. copemani* G1 and G2 parasites and $1x10^{10}$ *C. fasciculata* cells from the stationary phase were harvested by centrifugation at 14,000 rpm/4°C for 5 minutes and then washed once with PBS and once with NET100 buffer (10mM tris-Cl pH: 8, 100mM

NaCl, 100mM EDTA pH: 8). The pellet containing trypanosomes was submitted to lysis by re-suspending it in NET100 buffer and 1mg/ml of proteinase K. Sodium Sarkosinate (Sarcosyl) was added slowly (3% final concentration) and the suspension was gentle mixed and incubated for 30 minutes at 4°C. After this time, 24ml of CsCl (1.386 density) and 4ml of CsCl (1.750 density - mixed previously with 20µl of EtBr) were added to an ultra-clear SW28 rotor tube. 10ml of sample lysate was then added to the tube and centrifuged in a SW28 rotor at 20,000rpm/ 4°C for 30 minutes. The upper phase of the suspension was discarded and the middle phase (fluorescent band under UV light: KDNA-EtBr) was collected. The EtBr was removed by several washes with isoamyl-alcohol (0.1X SSC saturated) and then dialyzed overnight against tris EDTA buffer (TE) at 4°C. KDNA was finally purified using proteinase K (as above) followed by and phenol-chloroform extraction and ethanol precipitation.

7.3.4 Universal Minicircle Sequence PCR and sequencing

DNA from both *T. copemani* strains and *T. cruzi* Tulahuen strain was isolated using the DNeasy Blood & Tissue MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Because of the lack of sequence data of the minicircles from any Australian trypanosome on GenBank and the absence of designed primers to amplify them, this study attempted to amplify the minicircles of *T. copemani* using the 12-mer Universal Minicircle Sequence (5'-GGGGTTGGTGTA-3') that is conserved between the minicircles of most kinetoplastids and should be present in *T. copemani* as a forward primer (UMSF 5'-GGGGTTGGTGTA-3'), and its complementary sequence as a reverse primer (UMSR 5'-TACACCAACCCC-3'). PCR reactions were performed in a total volume of 25 µl containing 0.2 U of Taq DNA polymerase, 200 µM of dNTPs,

1.5 μM of $MgCl_2$, 1 μl of DNA template, and 1.5 μM of each primer. The primer concentration used was higher than the normally used (0.8 μM) in order to have enough primers annealing to target DNA, despite the competition of self-annealing. Amplification was performed in a PT100 thermocycler (MJ-Research) and consisted of an initial step of 95° C for 5 min, followed by 35 cycles of 30 s at 95° C, 30 s at 40° C and 60 s at 72° C and a final extension step at 72° C for 5 min. PCR products were run on a 1% agarose gel stained with SYBR safe (Invitrogen, USA), and visualised with a dark reader trans-illuminator (Clare Chemical Research, USA). Bands were cut from the gel and purified using Agencourt AMPure PCR Purification system (manufacturer's instructions) and sequenced using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Bio-systems, California, USA) on an Applied Bio-system 3730 DNA Analyzer. Sequences were aligned using MUSCLE (Edgar. 2004) with sequences from the minicircles of *T. cruzi* CL and Y strains retrieved from GenBank.

7.3.5 Cell lysates and Western blot analysis

Different concentrations of *T. copemani* G1 and G2, *T. cruzi* and *C. fasciculata* ranging from $20x10^6$ to 80 $x10^6$ parasites were collected and centrifuged at 14,000 rpm for 5 minutes at room temperature. The pellet was washed once with PBS and then resuspended in 40 μL of double-distilled water (H_2Odd). Then, 10 μL of Sodium dodecyl sulfate (SDS 10%) was added and samples were sonicated for 5 minutes. After sonication, cell lysates were solubilised in cracking buffer containing final concentrations of 50 mM Tris-HCl, pH 6.8, 4% (wt/vol) SDS, 3.5% (vol/vol) β-mercaptoethanol, 10% (vol/vol) glycerol, and 10 mM EDTA. The suspensions were then boiled at 100^oC for 5 minutes and loaded onto a 16.5% Tris-tricine SDS-polyacrylamide gel. Upper electrophoresis buffer was 0.1 M Tris-tricine, pH 8.2,

containing 0.1% SDS; lower buffer was 0.2 M Tris-HCl, pH 8.9. Protein bands on the gel were transferred onto nitrocellulose membranes (Schleicher and Schuell). Membranes were incubated with Ponceau staining for 5 minutes to confirm that protein bands were transferred to the nitrocellulose membrane, and then were washed with H_2Odd for several times. After this, membranes were blocked by incubation in 5% skim dry milk (Difco) diluted in PBS-Tween for 30 min, and then were probed with a 1: 4,000 dilution of anti-UMSBP for 90 min and with a 1:10,000 dilution of HRP-conjugated goat anti–rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) for 40 min, followed by ECL detection as recommended by the manufacturer (Amersham Pharmacia Biotech).

7.3.6 DNA Topoisomerase II assay

It has been demonstrated that minicircles within the kDNA network of trypanosomatids such as *C. fasciculata* are catenated and can be released from the network through decatenation by the enzyme DNA topoisomerase II (Shapiro, and Englund. 1995). In attempts to investigate if the minicircles of *T. copemani* were catenated into the kDNA network, the enzyme DNA topoisomerase II (TopoGen Inc, Port Orange, Florida, USA) was used. The 10 μl final volume reactions containing 4 units of DNA topoisomerase II, 50 mM Tris-Cl (pH 8), 120 mM KCl, 10mM $MgCl_2$, 0.5 mM of dithiothreitol (DTT), 0.5 mM of ATP and 30 μg BSA/ml (Topo II reaction buffer TG4040) and 1 μL of kDNA were incubated for 60 min at 37° C, and then stopped by the addition of 0.1 volume of stop buffer (5% sodium lauroyl sarcosinate, 0.025% bromophenol blue, 50% glycerol). Samples were loaded onto 1% agarose gel, containing 1μg/ml ethidium bromide (Life Technologies) and electrophoresed at 100 volts for 30 minutes.

C. fasciculata topo II decatenated kDNA and *C. fasciculata* linear kDNA markers were purchased (TopoGen Inc, Port Orange, Florida, USA) and used as controls in the assay.

7.4 Results

7.4.1 Kinetoplast morphology

The kinetoplast of both *T. copemani* strains exhibited the classical disk-shaped conformation present in other trypanosomatids such as *T. cruzi*, *T. brucei*, *Leishmania* and *C. fasciculata* (Shapiro, and Englund. 1995). It measured about 0.8 μm in length and about 0.24 μm in thickness, and was positioned adjacent to the basal body of the flagellum (Figure 1). Within the kinetoplast, the kDNA network was organised in the form of a giant and condensed disk-like structure. It was extended through the length of the kinetoplast (0.8 μm in length) and exhibited a thickness of approximately 0.19 μm (Figure 2).

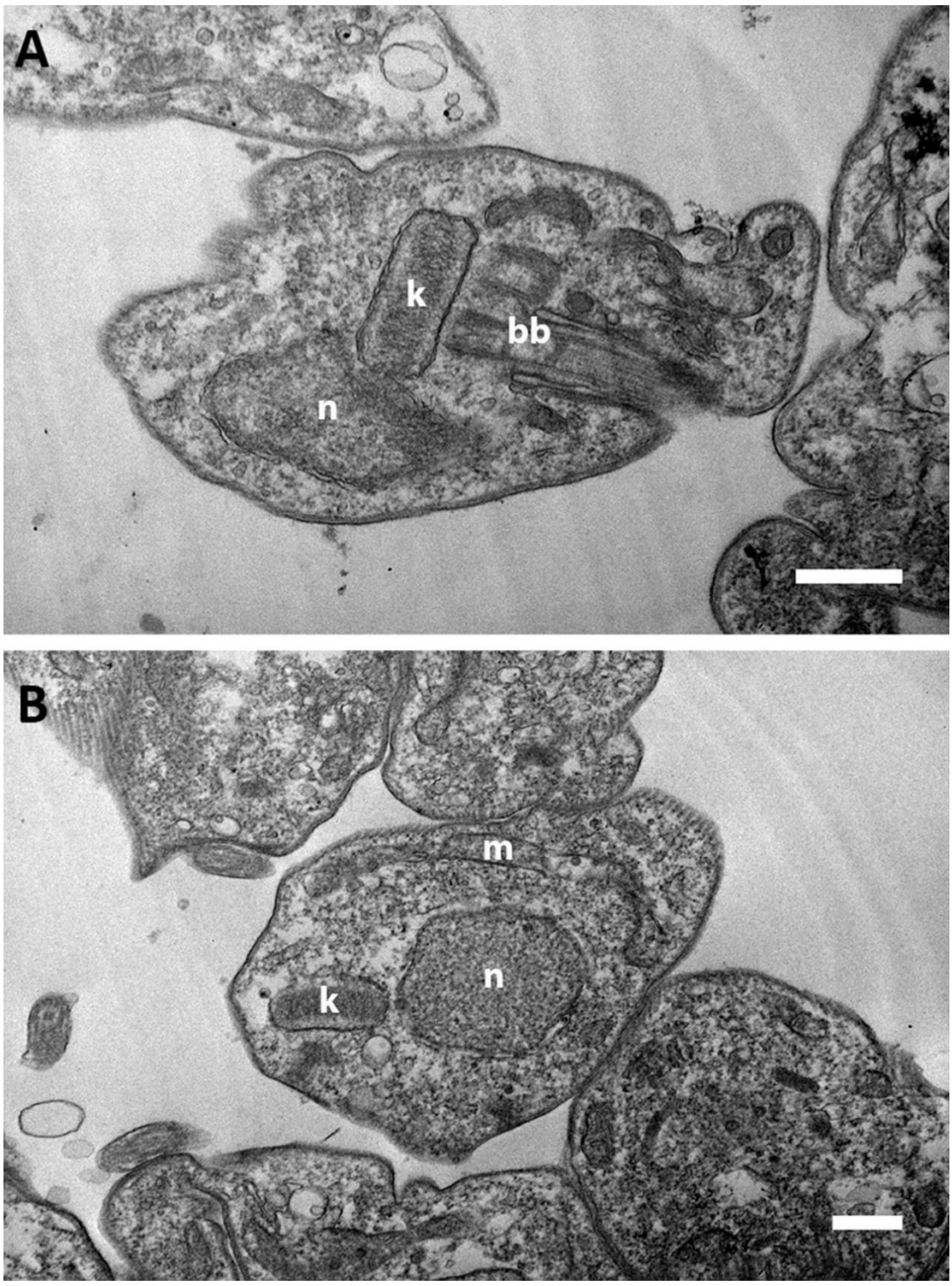


Figure 1. Electron microscopy cross-sections of *T. copemani* showing a disk-shaped kinetoplast. A: *T. copemani* G1, B: *T. copemani* G2, k: kinetoplast, n: nucleus, bb: basal body of the flagellum, m: mitochondrion. Scale bars: 0.5 µM.

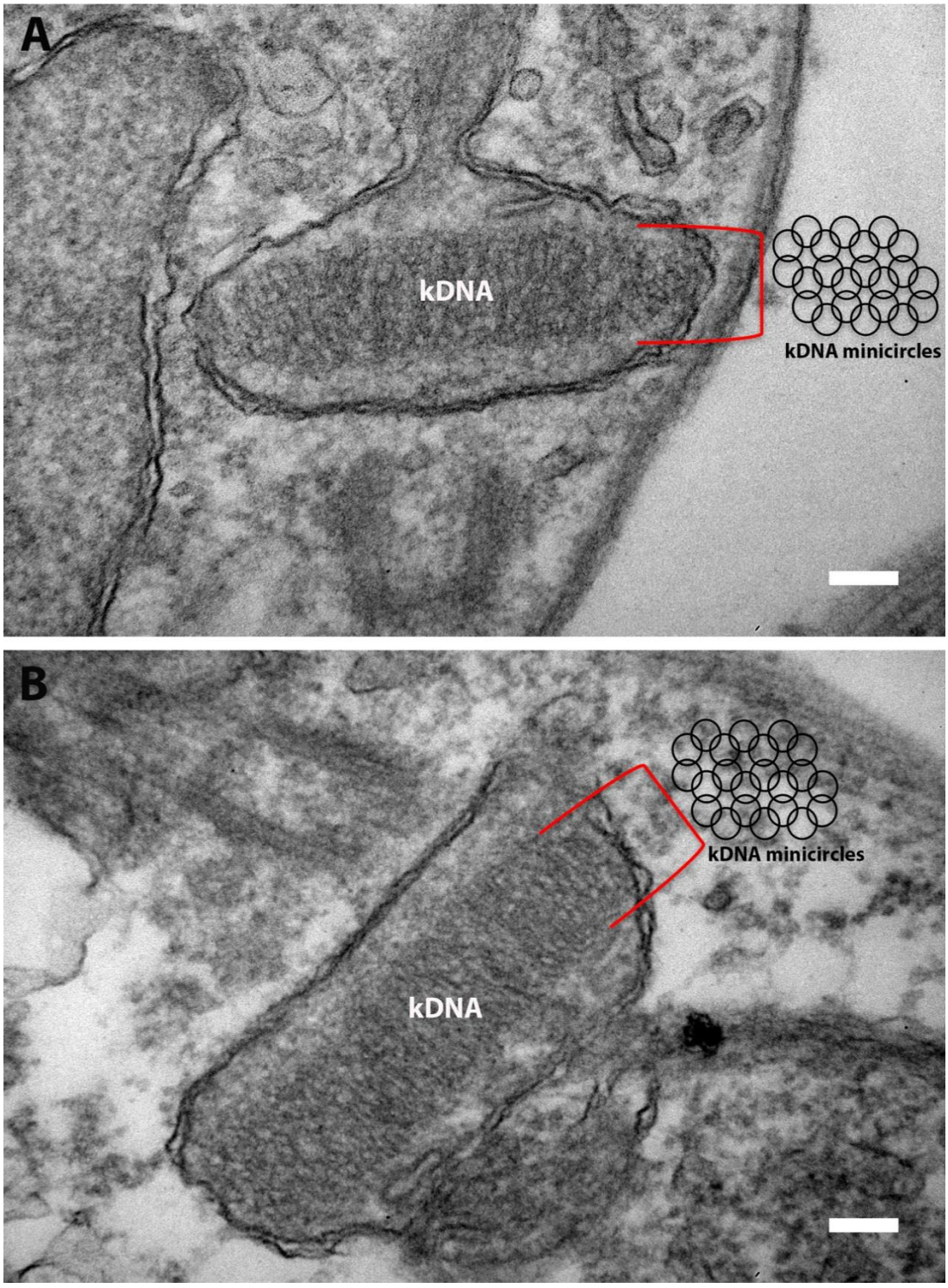


Figure 2. Electron microscopy cross-sections of the kDNA network of *T. copemani* and a schematic representation of minicircles organization within the network. A: kDNA of *T. copemani* G1, B: kDNA of *T. copemani* G2. Scale bars: 0.1 µM.

7.4.2 Universal Minicircle Sequence PCR and sequencing

A gradient PCR using different annealing temperatures and $MgCl_2$ concentrations was used initially to find the optimal conditions to amplify the variable regions of the minicircles using the UMS element as a forward primer and its complementary sequence as a reverse primer. When the PCR optimal conditions (described in materials and methods) were used, a band of approximately 300 bp was seen in both *T. copemani* G1 and G2 samples (Figure 3).

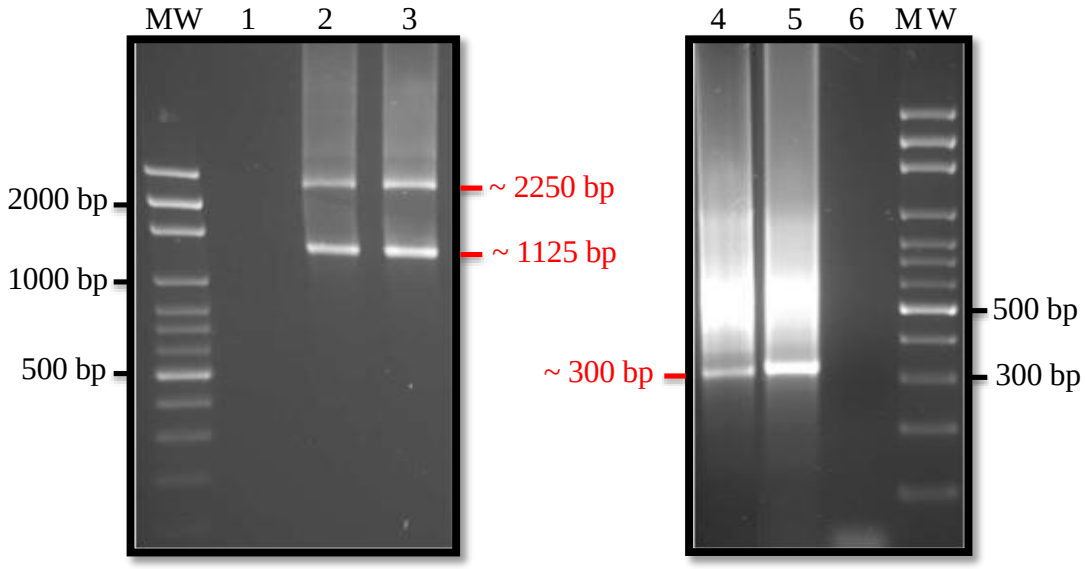


Figure 3. UMS-PCR products on agarose gels. MW: molecular weight markers. Lane 1 and 6: negative controls. Lanes 2 and 3: *C. fasciculate*, lane 4: *T. copemani* G1, lane 5: *T. copemani* G2, lanes 1 and 6: PCR negative controls, MW: molecular weight marker.

However, when the annealing temperature was decreased from 40° C (optimal temperature) to 38° C, additional diffuse bands of approximately 600 bp, 900 bp, and 1200 bp where observed (Figure 4). In contrast, when DNA of *C. fasciculata* and

T. cruzi were used as templates in the PCR, two bands of approximately 1,225 bp and 2,250 bp were seen for *C. fasciculata* (Figure 3), and four bands at approximately 350 bp, 700 bp, 1000 bp and 1400 bp were observed for *T. cruzi* (Figure 4).

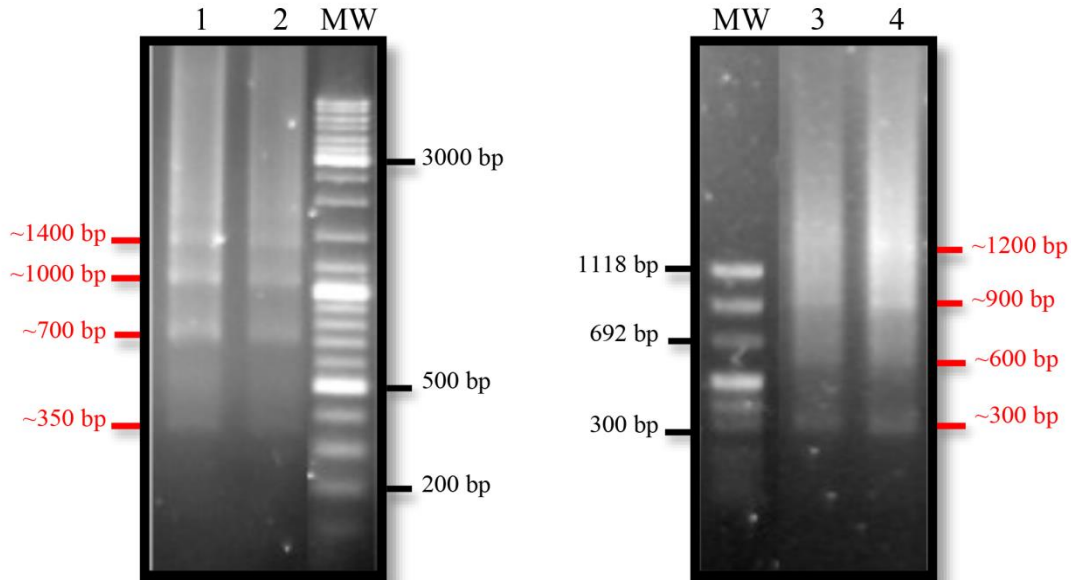


Figure 4. UMS-PCR products on agarose gels. Lane 1 and 2: *T. cruzi*, lane 3: *T. copemani* G1, lane 4: *T. copemani* G2, MW: molecular weight markers.

Interestingly, previous studies have shown the presence of two conserved regions in the minicircles of *C. fasciculata* and the presence of four conserved regions in the minicircles of *T. cruzi* (Sugisaki, and Ray. 1987b, Degrave *et al*. 1988) suggesting that the PCR amplifications obtained for both *C. fasciculata* and *T. cruzi* might be from the minicircles. The fact that the UMS-PCR exhibited four bands when *T. copemani* (either G1 or G2) was used suggests the presence of four UMS motifs in each minicircle (like in *T. cruzi*) and a full size of approximately 1200 bp as shown in Figure 5.

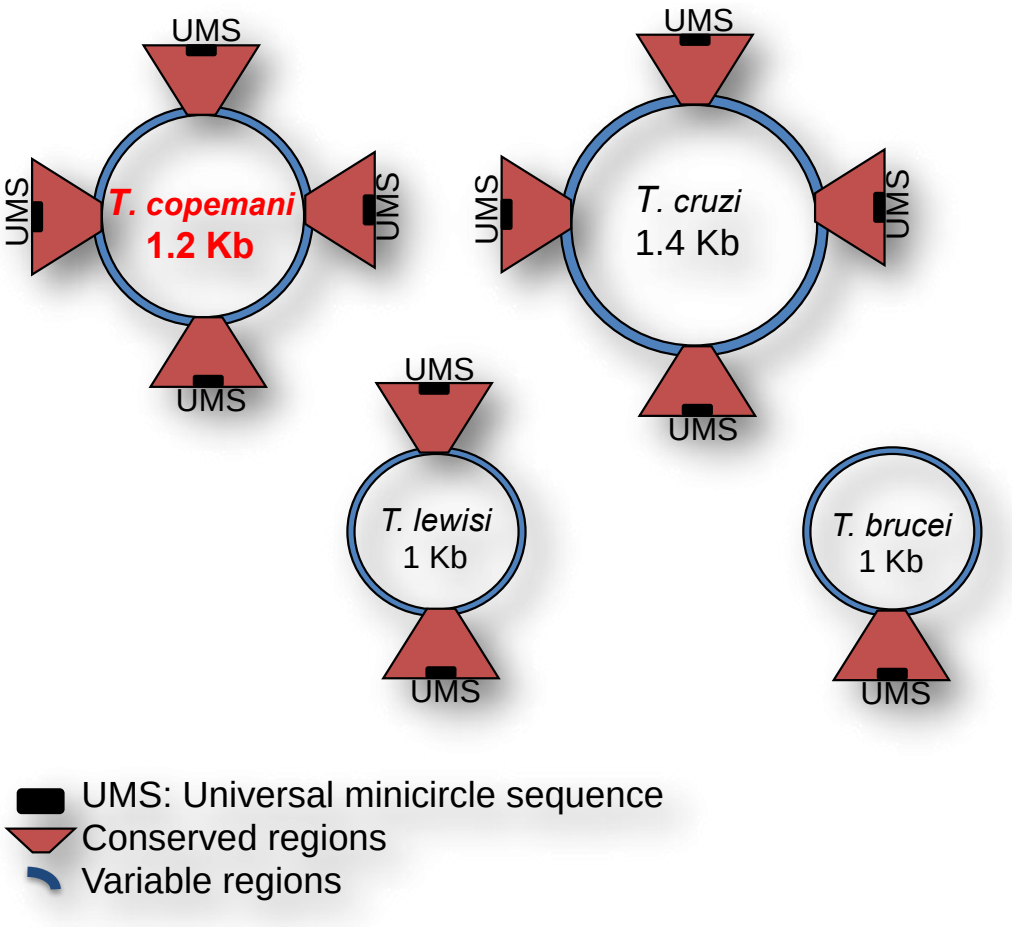


Figure 5. Proposed schematic representation of the position of the UMS elements in the minicircles of *T. copemani.* It also illustrates the structure of the minicircles of *T. cruzi*, *T. lewisi* and *T. brucei* and the position of the four, two and one UMS elements in the minicircles of each species respectively.

Sequencing of the PCR products confirmed they corresponded to the minicircles of *C. fasciculata* and *T. cruzi.* The *C. fasciculata* sequence was 431 bp long and when blasted to the NCBI database the closest related kinetoplastids was *C. fasciculata* clone M13CFK120 minicircle, with 92% of identity within 94% of query coverage. *T. copemani* G1 and G2 sequences were approximately 270 bp long and exhibited 27% of heterogeneity between them, with the majority of the heterogeneity in the first part of the sequence. Both sequences were blasted to the NCBI database and the closest related

kinetoplastid was *T. cruzi* with an 85% similarity within 44% of query coverage - most of the identical positions were at the downstream end (approximately 100 bp) (Figure 6). Alignments with the minicircles sequences of two strains of *T. cruzi* retrieved from GenBank revealed the presence of three conserved sequence blocks previously reported in all trypanosomatids (Table 1). The first and second blocks (CBS-1: 10 base pair sequence; CBS-2: 8 base pair sequence), which have been shown to differ between species, were exactly the same as the ones in both *T. cruzi* strain sequences. The third block CBS-3, which is the same UMS element (12 base pair sequence) was exactly the same as the one reported in several species of trypanosomatids (Table 1). Figure 6 shows the alignment of *T. copemani* G1 and G2 minicircle sequences with two sequences from the minicircles of the *T. cruzi* CL and Y strains.

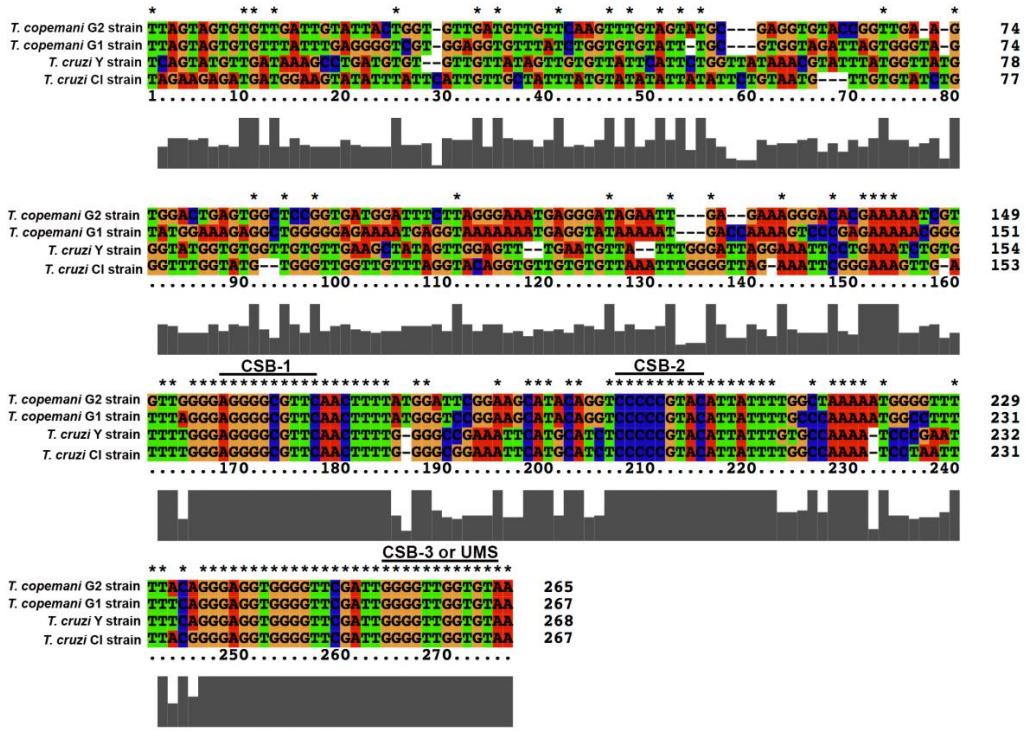


Figure 6. Alignment of the minicircles of *T. copemani* and *T. cruzi.* CSB-1, CSB-2 and CSB-3 or UMS: conserved sequence blocks between both *T. copemani* and *T. cruzi*.

Table 1. Conserved sequence blocks CSB-1, CSB-2 and CSB-3 (UMS) of different *Trypanosoma* species and *T. copemani*

Organism	CSB-1	D1-2	CSB-2	D2-3	CSB-3 or UMS	UMS per minicircle	Sequence Reference
T. brucei	ATGGGCGTGC	**20**	TCCCGTGC	**41**	GGGGTTGGTGTA	**1**	(Jasmer *et al*. 1986
T. congolense	AAGGGCGTTC	**29**	TCCCGTAC	**47**	GGGGTTGGTGTA	**1**	(Nasir *et al*. 1987)
T. lewisi	GAGGGCGTTC	**29**	CCCCGTAT	**47**	GGGGTTGGTGTA	**2**	(Ponzi *et al*. 1984)
T. equiperdum	ATGGGCGTGC	**21**	TCACGTGC	**38**	GGGGTTGGTGTA	**1**	(Barrois *et al.* 1981)
***T. cruzi* Y strain**	AGGGGCGTTC	**28**	CCCCGTAC	**47**	GGGGTTGGTGTA	**4**	(González. 1986)
***T. copemani* G1**	AGGGGCGTTC	**29**	CCCCGTAC	**48**	GGGGTTGGTGTA	**4**	This study
***T. copemani* G2**	AGGGGCGTTC	**29**	CCCCGTAC	**48**	GGGGTTGGTGTA	**4**	This study

D1-2: average distance (in base pairs) between CSB-1 and CSB-2
D2-3: average distance (in base pairs) between CSB-2 and CSB-3 or UMS

7.4.3 Universal Minicircle Sequence Binding Protein (UMSBP)

Considering that the UMS element is present in *C. fasciculata* minicircles, and that it was also found in *T. copemani* minicircles (by PCR and sequencing), we investigated the presence of a UMSBP in *T. copemani* using antibodies raised against *C. fasciculata* UMSBP (Tzfati *et al*. 1995b). A preliminary staining with Ponceau dye to evaluate the transfer (blotting) efficiency of the proteins to the membrane, showed the same pattern of protein bands for both *T. copemani* G1 and G2, and a different pattern when compared with *T. cruzi* and *C. fasciculata* (Figure 7). Western blot analysis using *C. fasciculata* anti-UMSBP antibodies showed this antibody recognised two peptides of approximately 14 kDa and 25 kDa in both *T. copemani* G1 and G2 protein extracts. These bands were detected only when more than 30 x 10^6 *T. copemani* epimastigotes were loaded per lane in the SDS-PAGE gel (Figure 8).

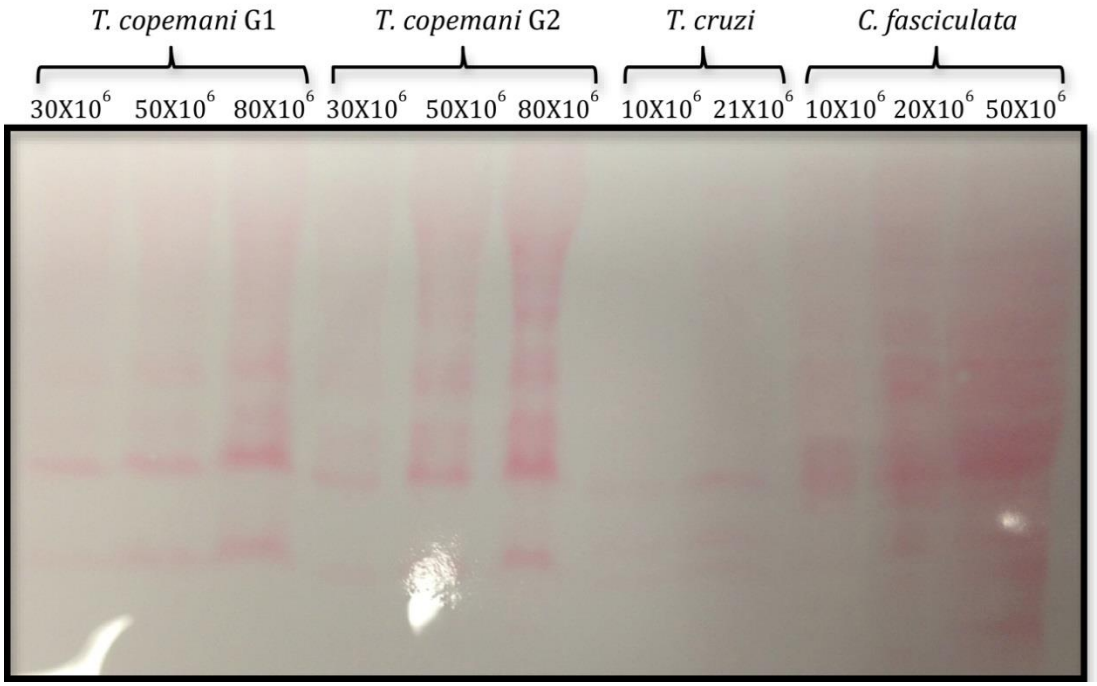


Figure 7. Nitrocellulose membrane stained with Ponceau dye for protein detection. Each lane contains different numbers of epimastigotes of *T. copemani* G1 and G2, *T. cruzi* and *C. fasciculata*.

When *C. fasciculata* protein extracts were used, the antibody recognised a peptide of approximately 13.7 kDa when all the three different concentrations of parasites were loaded per lane (Figure 9). However, when recombinant UMSBP from this trypanosomatid was used the antibody recognised an extra peptide of about 27.4 kDa. These results are consistent with previous studies that showed the UMSBP of this trypanosomatid is about 13.7 kDa and its dimer is double in size (27.4 kDa) (Tzfati *et al*. 1992, Tzfati *et al*. 1995b, Onn *et al*. 2004). When *T. cruzi* protein extracts were used, the antibody recognised a peptide of approximately 15 kDa (Figure 8).

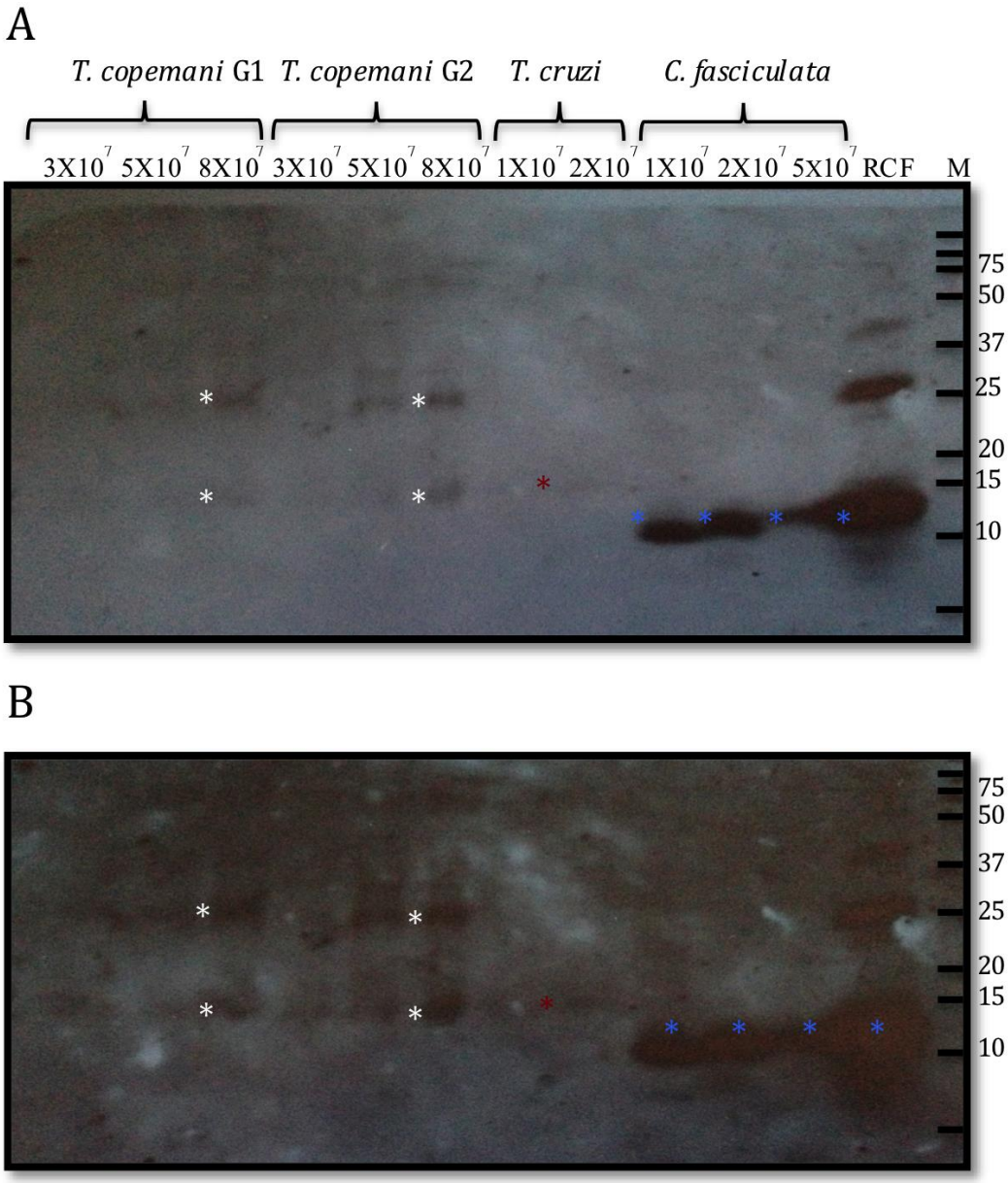


Figure 8. Western blot analysis using *C. fasciculata* UMSBP antibodies. Total protein cell extracts from different concentrations of epimastigotes of *T. copemani* G1, *T. copemani* G2, *T. cruzi* and *C. fasciculata* were used. Electrophoresis was carried out along with protein size marker (M); A and B: same membrane with different time of exposure; RCF: *C. fasciculata* recombinant UMSBP. White asterisks: *T. copemani* UMSBPs (~14 kDa and ~25 kDa). Red asterisks: *T. cruzi* (~15 kDa). Blue asterisks: *C. fasciculata* (~13,7 kDa).

7.4.4 KDNA decatenation

Incubation of purified kDNA with the enzyme DNA topoisomerase II revealed that the minicircles of *T. copemani* are catenated within the network. Released minicircles by topoisomerase II were analysed by electrophoresis in agarose gels. Intact kDNA networks where topoisomerase II was not added failed to enter the agarose gel (catenated minicircles). However, decatenated kDNA migrated into the gel and generated several bands (free minicircles released by topoisomerase II) (Figure 9).

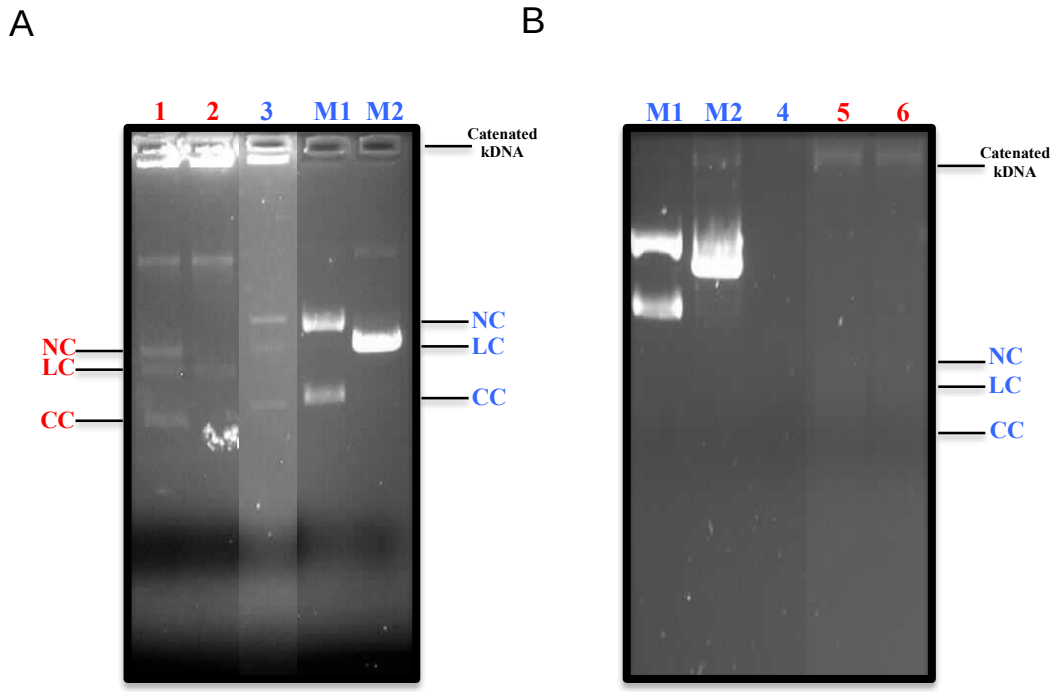


Figure 9. DNA Topoisomerase II products in *T. copemani* and *C. fasciculata* kDNA.

A: pure kDNA in presence of topoisomerase II (decatenation); B: pure kDNA without topoisomerase II (non-decatenation). In red: *T. copemani* kDNA. In blue: *C. fasciculata* kDNA and markers. Lanes 1 and 5: *T. copemani* G2. Lanes 2 and 6: *T. copemani* G1. Lanes 3 and 4: *C. fasciculata*. M1: *C. fasciculata* nicked open circular minicircles marker. M2: *C. fasciculata* relaxed minicircles marker. NC: decatenated nicked kDNA minicircles. LC: linearised kDNA minicircles. CC: decatenated covalently-closed minicircles.

Three bands were observed in the gel lane where topoisomerase II was added to kDNA of *C. fasciculata*. The upper and lower bands were equivalent to the bands in the decatenated kDNA marker (*C. fasciculata* marker) and corresponded to two decatenated products, nicked and covalently-closed minicircles respectively. The middle band was equivalent to the linear kDNA minicircles marker (*C. fasciculata* marker). In the same way, *T. copemani* G1 and G2 purified kDNAs yielded three bands, although, of smaller sizes when compared with *C. fasciculata* extracts. The upper and lower bands corresponded to nicked and covalently closed minicircles respectively. The middle band corresponded to linearised kDNA minicircles (Figure 9). The separation between the decatenated nicked and covalently-closed (relaxed) minicircles is due to the presence of EtBr in the gel, which results in the supercoiling of the covalently-closed, but not of the nicked minicircles. Topoisomerase II does not induce the formation of linear minicircle products suggesting that nicks possibly occurred during the purification of kDNA due to the presence of nucleases activity in the kDNA purification process. There was a high molecular weight band in all kDNA extracts that could be maxicircles or catenated minicircles (dimers, trimers, etc).

7.5 Discussion

Kinetoplastids are a group of flagellates that include Trypanosomatid parasites and free-living bodonid species. They have a mitochondrion termed a "kinetoplast" that contains DNA in two forms, known as maxicircles and minicircles. The organisation of minicircles and maxicircles within the kinetoplast DNA (kDNA) differs within species and these differences have been used to cluster them into various groups (Lukeš *et al*. 2002). The most complex type of kDNA organisation is the kDNA network structure. This type of kDNA is present in pathogenic trypanosomes such as *T. cruzi* and *T. brucei*,

and in the insect trypanosomatid *C. fasciculata* - all late diverging kinetoplastids (Lukeš *et al*. 2002). In the network, the kDNA is condensed into a disk-shaped structure where minicircles are covalently closed, and each is catenated on the average to three neighbours (Chen *et al*. 1995), and aligned side-by-side in a non-supercoiled structure (Rauch *et al*. 1993). Pro-kDNA is the second more organised kDNA structure and is present in the late-diverging free-living bodonid *Bodo saltans*. The majority of minicircles in pro-kDNA are covalently closed, topologically relaxed and organised in a single bundle-like structure with only a few catanenes (Blom *et al*. 2000). In contrast, all other types of kDNA structure, poly-kDNA, pan-kDNA and mega-kDNA are present in early diverging kinetoplastids such as *Dimastigella trypaniformis, Bodo caudatus, Cryptobia helicis,* and *Trypanoplasma borreli* (Lukeš *et al*. 2002). The kDNA is distributed from filling most of the kinetoplast such as Pan-kDNA, to filling various separate foci throughout the kinetoplast such as poly-kDNA, all lacking the highly ordered kDNA-packaging seen in trypanosomatids (Lukeš *et al*. 2002). Interestingly, the kinetoplast of *T. copemani* presented a classical kDNA network conformation similar to the one seen in late diverging kinetoplastids such as *T. cruzi* and *T. brucei*. Furthermore, topoisomerase II products demonstrated that the minicircles of this parasite are catenated, confirming that the arrangement of the kDNA of *T. copemani* is in the form of a catenated network as seen in *T. cruzi* and *T. brucei*. The shared features in the kinetoplast ultrastructure between late diverging kinetoplastids and *T. copemani* might be of significance from the point of view of the evolution of Australian trypanosomes. However, further studies are needed to better understand these relationships.

Not only does the kDNA organization differ between species, several studies have reported differences in size of minicircles, and also differences in the number and position of the UMS elements in each minicircle. The size of the minicircles of *C. fasciculata* and *T. lewisi* for example, are approximately 1 Kb and 2.5 Kb respectively. All minicircles of both species contain two UMS elements located 180 degrees apart (Ponzi *et al*. 1984, Sugisaki, and Ray. 1987b, Degrave *et al*. 1988). In contrast, *T. brucei* and *Leishmania tarentolae* minicircles, are about 1 Kb in size, and contain only one UMS element (Kidane *et al*. 1984, Jasmer, and Stuart. 1986, Hines *et al*. 2011). The results of this study are consistent with those previously published for *T. cruzi* and *C. fasciculata*. *T. cruzi* UMS-PCR showed the amplification of four bands of approximately 350 bp, 700 bp, 1000 bp and 1400 bp, suggesting that primers aligned at the UMS elements present at zero and 90 degrees amplifying a quarter of the minicircles; at zero and 180 degrees amplifying half of the minicircle; at zero and 270 degrees amplifying three quarters; and at zero and 360 degrees amplifying the full minicircle (Figures 4 and 5). In contrast, when *C. fasciculata* kDNA was used, two bands of approximately 1125 bp and 2250 bp were obtained suggesting that primers aligned either in the UMS located at zero and 180 degrees amplifying half of the minicircles, and at zero and 360 degrees amplifying the full minicircles (Figures 3 and 5). Interestingly, when *T. copemani* kDNA was employed, the amplification of four bands slightly smaller than those obtained with *T. cruzi* were seen. The size of the four bands doubled in size (300 bp - 600 bp - 900 bp -1200 bp) suggesting the presence of four UMS elements (conserved regions) located at zero, 90, 180 and 270 degrees in each minicircle, and a full size of the minicircles of *T. copemani* of approximately 1200 bp (Figures 4 and 5). Although the use of a pair of complementary short primers (like the 12-mer UMS primers used in this study) is not ideal due to possible nonspecific and

self-annealing amplifications, the lack of sequence data of the minicircles of *T. copemani* in GeneBank and the lack of any primers to amplify them made these short sequences the only options to explore. Supporting the PCR results, sequencing of the UMS-PCR products confirmed that the regions amplified by these primers corresponded to minicircle DNA. Similarly, the use of the UMS element as a forward and reverse primer to amplify the minicircles of *Herpetomonas samuelpessoai* was demonstrated previously (Fu *et al*. 1999).

As previously discussed, the thickness of the kinetoplast disk varies among different species and is about half the circumference of a minicircle (Simpson. 1972). In *T. carassii* for example, the kDNA network disk thickness is about 0.233 µM and its minicircles are 1.6 Kb (Lukeš *et al*. 2000). Assuming this demonstrates correlation between the kDNA disk thickness and the size of minicircles, the suggested size of the minicircles of *T. copemani* of around 1.2 Kb seems to be correct. However, further cloning and sequencing of the full length of the minicircles of *T. copemani* is required to verify this.

The sequence regions flanked by the UMS elements have been shown to differ at the intraspecific level. In *T. cruzi* for example, these variable regions have been used as powerful markers to discriminate between different stocks or strains using minicircle restriction fragment profiles (Morel *et al*. 1980), low stringency single specific primer PCR (LSSP-PCR) (Rodríguez *et al*. 2009), and Southern hybridization analysis using labelled minicircles variable regions (Botero *et al*. 2010). Sequencing of the regions amplified by the UMS element primers showed a high heterogeneity between both strains of *T. copemani* and both strains of *T. cruzi* at the beginning of the sequence (first

150 bp). Most likely, this region corresponds to a portion of the variable regions of the minicircles of both parasites. In contrast, a low heterogeneity in sequence was observed at the end of the sequence (last 100 bp). This region contained the three conserved sequence blocks CSB1, CSB2, and CSB3 or UMS, and therefore may correspond to the conserved region of the minicircles.

When sequences were individually compared with those in GenBank, the closest match were minicircle sequences of *T. cruzi* with 83% of identity in the last 138 nucleotides of the sequence (half of the sequence). When this more conserved part of the sequence between both species was analysed more carefully, three conserved sequence blocks were found in both species including the UMS element block (which is also known as CSB-3). Those three conserved sequence blocks have been reported previously from different species of trypanosomatids including *C. fasciculata*, *L. tarentolae*, *T. brucei*, *T. congolense*, *T. equiperdum* and *T. lewisi* (Barrois *et al*. 1981, Kidane *et al*. 1984, Ponzi *et al*. 1984, Jasmer, and Stuart. 1986, Nasir *et al*. 1987, Sugisaki *et al*. 1987b) and are believed to be important for DNA replication (Shapiro *et al*. 1995). However, differences in the sequence of both first and second blocks (CSB-1 and CSB-2) between different trypanosomatids have been reported (Ray. 1989). Interestingly, *T. copemani* CSB-1 and CSB-2 block sequences were exactly the same as the ones seen in different clones of *T. cruzi* minicircles. The fact that CSB-1, CSB-2 and CSB-3 minicircle blocks are involved in the initiation of kDNA replication and all were identical in both *T. cruzi* and *T. copemani* suggest that both parasites might share common features in the process of kDNA replication. However, the significance of these genetic similarities and this assumption needs to be further investigated.

The UMSBP of *C. fasciculata* has been extensively studied. This protein is a single-stranded DNA-binding protein that has been thought to act as an initiator in the minicircles replication process (Milman. 2007) after binding to the UMS elements in the minicircles (Tzfati *et al*. 1992, Tzfati *et al*. 1995b). It has been shown that antibodies raised against the UMSBP of *C. fasciculata* can cross react with the UMSBP of other trypanosomatids such as *T. cruzi* and *T. brucei* (Coelho *et al*. 2003, Milman. 2007). *T. cruzi* UMSBP was previously shown to be approximately 14 kDa and to bind to the dodecamer UMS element (Coelho *et al*. 2003). The present study confirmed these previous results and showed that antibodies raised against *C. fasciculata* UMSBP recognise *T. cruzi* UMSBP as well. However, there was a slight difference in the size of the protein (approximately 15 kDa in this study). This study reports for the first time the presence of UMSBP in *T. copemani.* Antibodies raised against the UMSBP of *C. fasciculata* detected two proteins in *T. copemani* cell extracts, suggesting the presence of two UMSBP orthologues in this parasite, of about 14 and 25 kDa each. The identification of *T. copemani* UMSBP, a protein that has been involved in the process of kDNA replication in other trypanosomes, is the first step to understanding this mechanism in this parasite. It has been demonstrated that knocking down the *UMSBP* gene *in vitro*, not only affects the initiation of minicircle replication, but also inhibits segregation of the daughter networks and stops nuclear division and caused cell growth arrest (Milman *et al*. 2007). Considering the observation that *T. copemani* was associated with the drastic decline of the woylie and knowing the UMSBP of this parasite, open the doors to the development of new drug candidates that target this indispensable protein in the pursuit of strategies to reduce the impact of *Trypanosoma* infections within Australia.

This study demonstrated not only strong similarities in the kDNA structure and minicircle organisation and sequence between *T. copemani* and late emerging trypanosomatids such as *T. cruzi*, but it also provided preliminary information and a foundation to better understanding the minicircle organisation and the mechanism of kDNA replication in Australian trypanosomes.

7.6 References

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Chapter 8

General discussion

8.1 Summary

This chapter reviews the findings of this whole research and links the different chapters in the context of the overall aims. First, it describes the aims and summarises the methodology used to accomplish the genotypic and phenotypic characterisation of Australian trypanosomes. Secondly, it briefly describes the main results of each chapter and emphasises the importance of the overall findings. Finally, it considers the findings in a broad perspective and suggests directions for future research.

8.2 General overview

The overall aim of this research project was to investigate the genotypic and phenotypic diversity of *Trypanosoma* spp. infecting Western Australian marsupials and to determine the potential role that trypanosomes are playing in the drastic decline of the woylie. On one hand, the genotypic characterisation involved the sequencing and phylogenetic analysis of trypanosomes in the blood and tissues of ten different marsupial species using two different loci: the nuclear 18S rDNA and the mitochondrial g*GAPDH* gene, as well as the sequencing of partial fragments of the minicircles of the kinetoplast DNA of trypanosomes isolated in culture. On the other hand, the phenotypic characterisation involved different aspects of trypanosome biology and behaviour including: (i) investigation of trypanosomes capacity to migrate to different tissues in naturally infected marsupials; (ii) description of the different morphological stages of trypanosomes in the vertebrate host and in culture media; (iii) investigation of trypanosomes capacity to infect different cell lines *in vitro* (iv) evaluation of their susceptibility to different drugs *in vitro*; and finally (v) the description of the ultrastructure of their kinetoplast.

The core of both genotypic and phenotypic characterisation was the critically endangered Australian marsupial "the woylie", or brush-tailed bettong, *Bettongia penicillata,* which interestingly was the only marsupial host infected with all eight different *Trypanosoma* genotypes described, and from which trypanosomes were isolated in culture. The drastic population decline - where disease is believed to be playing an important role, and the high prevalence of *Trypanosoma* infections in the woylie, made this marsupial species the perfect candidate to investigate the potential pathogenicity of Australian trypanosomes. This study also provided a unique biological comparison of Australian trypanosomes with the pathogenic *T. cruzi* in order to better understand the potential pathogenicity of Australian trypanosomes.

The main results of each chapter are briefly summarised as following:

***Chapter 1*:** This chapter comprised a review of different aspects of trypanosome life history including evolution, genetic diversity, taxonomy, host range, life cycle, pathogenicity, and drug efficacy.

***Chapter 2*:** This chapter described the presence of eight different genotypes belonging to three different species of indigenous Australian trypanosomes (*T.* sp H25, *T. copemani* and *T. vegrandis*) circulating in blood and tissues of ten different species of marsupials and revealed a remarkably high prevalence of trypanosome infection in the critically endangered marsupial the woylie.

***Chapter 3*:** This chapter demonstrated a high prevalence of infection with *T. copemani* in woylies in the declining population and a high prevalence of *T. vegrandis* in woylies in the stable population. It showed also that woylie tissues infected with *T. copemani* G2 or co-infected with some genotypes of *T. vegrandis* and *T.* sp H25 exhibited

pathological lesions similar to those seen in infections with the pathogenic *T. cruzi*, suggesting an association between *Trypanosoma* infections and the drastic decline of the woylie.

***Chapter 4*:** This chapter described the isolation and morphology of trypanosomes, and showed differences in the nutritional requirements and behaviour in culture of *T. copemani* G1 and G2.

***Chapter 5*:** This chapter revealed that *T. copemani* G2 was able to infect different cell lines *in vitro*, and was able to produce a higher infection in *VERO* cells than the pathogenic *T. cruzi*.

***Chapter 6*:** This chapter demonstrated remarkable differences in drug sensitivity between *T. copemani* G1 and G2 and showed that drugs currently used to combat *T. cruzi* infections are effective against these parasites.

***Chapter 7*:** This chapter showed outstanding similarities between the organisation and sequence of the minicircles of the kinetoplast DNA of *T. copemani* and *T. cruzi.*

Importance of the results

Understanding the life cycle of parasites is essential in the development of strategies to reduce the infection pressure generated in the host. The findings of this PhD study provided novel and unique insights into the capacity of Australian trypanosomes to migrate and infect different tissue cells in the vertebrate host, greatly advancing the understanding of the life cycle of these parasites within Australian wildlife. The similarities found in the life cycle of *T. copemani* G2 and *T. cruzi* has important implications for the pathogenicity of these trypanosomes and conservation of marsupial species where this Australian trypanosome is known to be at high prevalence levels.

Together all the data presented in this study helped to clarify the high genetic diversity of Australian trypanosomes and provided new evidence for the potential role of trypanosomes in the decline of a formerly abundant marsupial that is now critically endangered, the woylie. The results not only contributed valuable information towards directing management decisions for endangered species where these parasites are known to be present at high prevalence levels, but also provided new knowledge about the evolutionary biology and relationship that Australian trypanosomes have to the exotic and pathogenic *T. cruzi*. The information gained on host-parasite associations and pathogenicity will be significantly informative in fauna translocation planning and captive breeding.

The data generated provided a framework for future research into developing strategies for limiting the risk of infection and spread of trypanosomes within Australian wildlife, and invites to the investigation of the role that these infections are playing in the decline of other native marsupials that are threatened or endangered.

8.3 Perspectives and future directions

8.3.1 The Isolation of Australian trypanosomes in culture

The investigation of parasite biology and host-parasite interactions cannot be fully understood without the isolation of parasites in culture. The fact that some trypanosomes have been isolated in culture has facilitated not only a full understanding of their life cycle, but also the biological mechanisms underlying host-parasite interactions such as virulence and pathogenicity.

Unfortunately, culture-based methods tend to miss much of the diversity of trypanosomes in nature due to the wide and different spectrum of nutritional conditions required among *Trypanosoma* species, and finding the most effective culture medium that contains all the nutrients required for growth of certain species can be very difficult. Trying to overcome this problem, and taking into account the huge diversity of trypanosomes circulating in Australian marsupials, this study used different culture media in attempts to isolate all genetically distinct trypanosomes infecting Western Australian marsupials. Although, several isolates were successfully established in culture, PCR and sequencing showed all of them were *T. copemani* G1 or G2. The isolation of *T. copemani* G1 and G2 in culture allowed the investigation of diverse phenotypic characteristics including morphology, growth kinetics, cell infection, drug susceptibility, and kinetoplast DNA organisation, thus considerably increasing our knowledge about the biology of these parasites. The isolation of these trypanosomes in culture also opened doors for future *in vitro* and *in vivo* investigations towards understanding the mechanisms they employ for cell invasion and replication, and thus better defining their pathogenic potential.

Although several different media were used, neither *T. vegrandis* or *T.* sp H25 were established in culture suggesting that the nutritional growth conditions required by both species might not be supplied by the different media used. However, the difficulty in isolating *T.* sp H25 could also be reflected by the low prevalence of this parasite in all marsupials examined. It is important to isolate *T. vegrandis* and *T.* sp H25 in culture, not only because they have been found infecting several tissues of various threatened and endangered marsupial species (Botero *et al*. 2013), but also because pathological signs were seen in woylie tissues co-infected with both species.

Polyparasitism is a term commonly used when referring to concurrent, mixed or co-infections of either different species and/or intraspecific parasite genotypes (Lymbery *et al*. 2012). Mixed infections involving different species or genotypes of trypanosomes occur frequently in nature (Martins *et al*. 2006, Pollitt *et al*. 2011, Charles *et al*. 2013), and their interaction in a single host can influence their life history traits and dynamics in natural infections (Reifenberg *et al*. 1997). In *T. cruzi* for example, natural infections are composed of different genotypes of the parasite (multiclonal infections) (Tibayrenc *et al*. 1986, Tibayrenc *et al*. 1988), with each genotype exhibiting a preferential tissue tropism (Andrade *et al*. 1999, Vago *et al*. 2000, Macedo *et al*. 2004, da Silva Manoel-Caetano *et al*. 2008, Ramírez *et al*. 2010). Charles *et al*. (2013) showed that Southern plains woodrats (*Neotoma micropus*) could be co-infected with two genotypes of *T. cruzi* and a novel *Trypanosoma* species. Moreover, polyparasitism involving two different species, *T. cruzi* and *T. rangeli,* has been reported in the critically endangered species of tamarin, *Saguinus bicolor* (Maia da Silva *et al*. 2008).

Considering that polyparasitism with different species and genotypes of trypanosomes were prevalent and associated with pathology in tissues from woylies in the declining population there is a need to better understand if the presence of competing genotypes or species in a single host can modify the infection dynamics by either reducing or enhancing parasitemia, virulence, and pathogenicity. The first step to understand this will be to perform controlled *in vitro* and *in vivo* laboratory mixed infection experiments that can only be achieved with the isolation of both *T.* sp H25 and *T. vegrandis* in culture. Developing and using two/three-trypanosomes in co-infection models will assist in identifying if the competition for resources *in vitro* (with or without cells) or *in vivo* (in an experimental model) will select for higher or lower virulence trypanosomes.

Understanding if a species or genotype of trypanosomes in mixed infections reduces the virulence of others could be a clue to developing strategies to control trypanosomes that are causing problems in wildlife. The isolation of both *Trypanosoma* species in culture will also help to understand their life cycle, which can be particularly interesting for *T.* sp H25 that was shown to be genetically closely related with the pathogenic *T. cruzi*.

8.3.2 The remarkable genetic diversity within the Trypanosoma copemani and Trypanosoma vegrandis clades

Over the past two decades, the advent of molecular techniques has uncovered a large degree of genetic diversity within species of trypanosomes. Interestingly *T. copemani* and *T. vegrandis* clades exhibited a remarkably high intraspecific genetic variation, with ten and thirteen different genotypes respectively with polymorphisms at the 18S rDNA and g*GAPDH* genes described in both clades (including *T. copemani* G1 and G2 and *T. vegrandis* G3, G4, G5, G6 and G7 described in this study) (Austen *et al*. 2009, Paparini *et al*. 2011, Botero *et al*. 2013). Intraspecific genetic variation between trypanosomes has been attributed to genetic exchange, which has been widely described in *T. brucei* (Tait *et al*. 1990, Peacock *et al*. 2011), or to the multiclonal population structure described in *T. cruzi* (Tibayrenc *et al*. 1986). *T. brucei* for example, uses DNA recombination to periodically switch the expression of variant surface glycoprotein genes to evade the mammalian immune response in a process named antigenic variation (Hall *et al*. 2013). In contrast, a multiclonal population structure has been the explanation of the high intraspecific genetic diversity within *T. cruzi* (Tibayrenc *et al*. 1986, Valadares *et al*. 2012). However, recent evidence has demonstrated that genetic exchange events also occur in *T. cruzi* (Ramírez *et al*. 2013, Roellig *et al*. 2013), and

that it could be a possible source of the genetic diversity observed in *T. cruzi* sylvatic populations (Miles. 1996).

It is unclear at this time whether the genetic diversity observed within *T. copemani* and *T. vegrandis* is a product of either genetic exchange or multi-clonality and the fact that only some genotypes within both clades were found infecting tissues could be explained by both mechanisms. On one hand, genetic exchange might be yielding *T. copemani* or *T. vegrandis* genotypes with new combinations of biological properties such as virulence and pathogenicity. It was shown that under experimental conditions genetic recombination gave rise to more virulent trypanosomes. *T. b. rhodesiense* is pathogenic to humans due to the presence of a serum resistance associated gene, SRA, which prevents the lysis of the parasite by human serum. In contrast, *T. b. brucei* is not pathogenic to humans because it lacks the SRA gene (Tomlinson *et al*. 1998). However, under experimentally conditions, it was shown that the transfection of *T. b. brucei* with the SRA gene is sufficient to confer resistance to human serum (Van Xong *et al*. 1998). Furthermore, a recent study showed that the SRA gene could be gained by genetic recombination events in natural infections. Balmer *et al*. (2011) demonstrated that multiple infections enable the transference of the SRA gene from *T. b. rhodesiense* to *T. b. brucei* giving rise to new pathogenic strains. Thus, genetic exchange could be the explanation of the genetic diversity within both *T. copemani* and *T. vegrandis* clades, and might explain the appearance of some genotypes within each clade that are able to infect tissues and to cause pathology in the vertebrate host. On the other hand, it could also be possible that trypanosomes in natural infections are multiclonal with each clone presenting a different biological behaviour, some being able to establish better in host tissues and some being preferentially established in blood. However, the occurrence of

genetic exchange with a later clonal expansion of specific genotypes that become stable in the host could also be possible. To demonstrate that either genetic exchange and/or a multiclonal structure is accounting for the intraspecific genetic diversity observed within both *T. copemani* and *T. vegrandis* clades, additional studies involving the direct cloning and genotyping from single cells and their use in controlled mixed infections are necessary.

In addition, the lack of host specificity reflected by the capacity of all *Trypanosoma* species found in this study to infect several marsupial species could indicate that host-switching events might have contributed to recombination events, or to the appearance of multiclonal infections. The implications of the genetic diversity of Australian trypanosomes on virulence and pathogenicity needs to be further investigated, not only because of their capacity to adapt and exploit different hosts, but also because this plasticity can determine their successful ability to become established in new hosts after their introduction into new habitats.

8.3.3 Trypanosoma copemani G1 and G2

Combined genotypic and phenotypic data are fundamental when finding and delimiting new *Trypanosoma* species. The level of morphological and genetic heterogeneity that defines a *Trypanosoma* species is not yet established. However, data on the extent of variation within and between known *Trypanosoma* species that are genetically and biologically related could help in determining what constitutes a new species. This study found remarkable phenotypic differences between *T. copemani* G1 and G2, but at the genotypic level variation was insufficient to consider them as separate species. The genetic polymorphisms in both the 18S rDNA or gGAPDH sequences and the genetic

distances between both *T. copemani* G2 and *T. copemani* G1 (and other genotypes closely related to G1 - see chapter 2) were lower than the diversity seen between two closely related *Trypanosoma* species, *T. cruzi* and *T. marinkellei*.

New molecular markers such as cytochrome B and more biological differences must be investigated before definitive statements concerning *T. copemani* G2 as a separate species can be made. Furthermore, the development of primers that can discriminate between *T. copemani* G1 and G2 are necessary to better understand the dynamic of infection of both genotypes in a single host. This will also facilitate longitudinal studies looking at the prevalence of infection with both genotypes and comparing the health outcome of infections in wildlife. If *T. copemani* G1 and G2 are the same species, further studies will be needed to identify the parasite key mechanisms involved in their diverse phenotypic behaviour, for example the genes determining *T. copemani* G2 virulence and pathogenicity. Knowledge of these genes could provide insights into the understanding of the evolution of pathogenicity in Australian trypanosomes.

8.3.4 The Australian Trypanosoma copemani G2 and the South American Trypanosoma cruzi

Both *T. copemani* G2 and *T. cruzi* have been shown to cause pathology in vertebrate host tissues. Like the pathogenic *T. cruzi*, the Australian *T. copemani* G2 migrates to different tissues in the vertebrate host and once there is able to infect cells, as demonstrated in natural infections in the woylie and in *in vitro* infections using different cell lines (see chapters 3 and 5). The significance of these findings has important implications for pathogenicity. Firstly, *T. copemani* G2 might infect cells to evade the host immune system and to complete its life cycle similar to *T. cruzi*. This may explain

the persistence of trypanosomes in the blood of naturally infected woylies that were examined consecutively for long periods of time (Thompson *et al*. 2014), and the presence of *T. copemani* G2 in host tissue cells (Botero *et al*. 2013). Secondly, *T. copemani* G2 might trigger an immune inflammatory response that results in tissue damage and necrosis of host tissues, similar to that seen in *T. cruzi* infections in marsupials and humans in South and North America. Although the phylogenetic analysis based in the 18S rDNA and g*GAPDH* sequences showed that *T. copemani* does not clade with *T. cruzi* (such as *T.* sp H25), sequence blocks from the conserved regions of the minicircles of the kDNA of both parasites were identical. The fact that these identical regions are involved in the initiation of kDNA replication suggests that both parasites might share common features in the process of kDNA replication.

The capability of both *T. copemani* and *T. cruzi* to infect cells suggests that these parasites might explore similar strategies to complete their life cycle in the vertebrate host (Figure 1 illustrates the proposed life cycle of *T. copemani* G2 in the vertebrate host). However, the use of a better *in vitro* model and an *in vivo* model will be necessary to confirm this. A marsupial cell line isolated from any Australian native marsupial known to be susceptible to *T. copemani* infections may be a good *in vitro* model to use. The use of a marsupial cell line in experimental infections with *T. copemani* G2 and *T. cruzi*, may contribute to better understanding the potential of *T. copemani* for intracellular replication and also may provide insights into the potential capacity of *T. cruzi* to infect Australian marsupials.

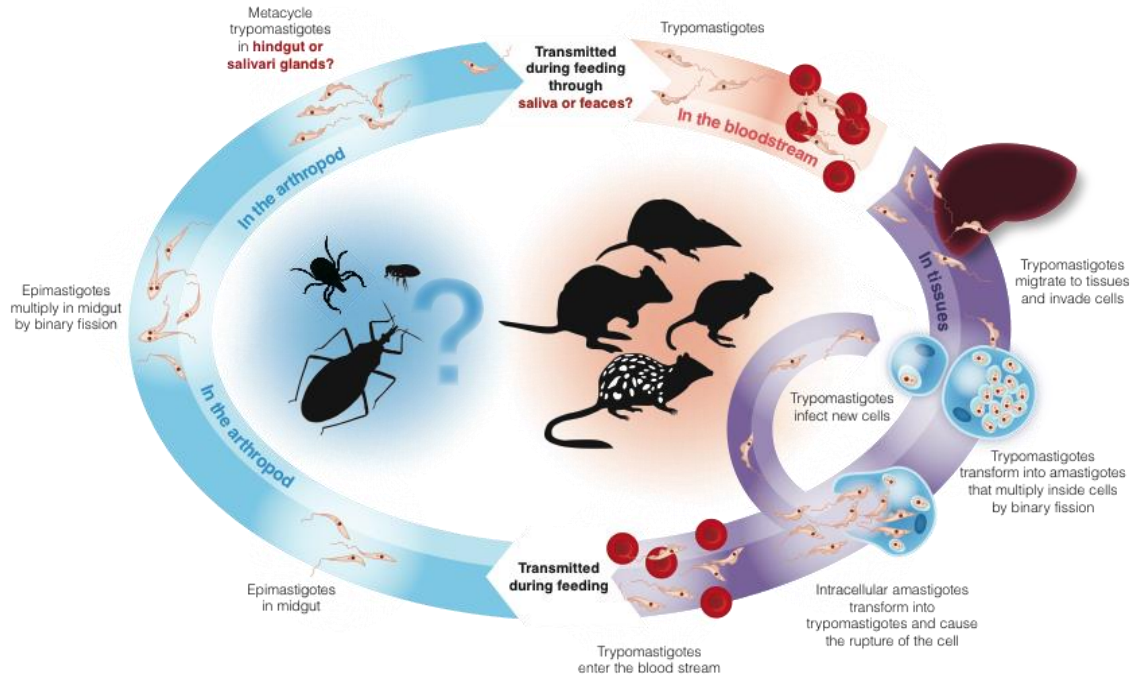

Figure 1. Proposed life cycle of *T. copemani* G2 in the marsupial host.

8.3.5 Australia's biosecurity

T. cruzi is the agent of Chagas disease in humans and also infects several marsupial species in South and North America, whereas *T. copemani* is a marsupial trypanosome that may not be capable of infecting humans. However, with human population growth and encroachment on wildlife habitats, the possibility exists that spillover events can occur within Australia (Thompson. 2013). It is noteworthy that some *Trypanosoma* species that normally are restricted to animals have been reported atypically infecting humans. These included infections with *T. b. brucei*, *T. vivax*, *T. congolense*, *T. evansi* and *T. lewisi*. While, some of these infections were transient in patients who recovered with or without treatments with anti-trypanosomal drugs, others caused illnesses and sometimes the death of patients with immature (infants) or depressed immune systems (Abebe *et al*. 1988, Truc *et al*. 1998, Joshi *et al*. 2005, Howie *et al*. 2006, Kaur *et al*.

2007, Sarataphan *et al*. 2007, Deborggraeve *et al*. 2008, Haridy *et al*. 2011, Verma *et al*. 2011). In the event that any case of human trypanosomiasis caused by *T. copemani* occurs in Australia, the drugs benznidazole and the fenarimol and pyrimidine derivatives, which this study found to be highly effective against this parasite, could be a potential treatment to be used.

Exotic vector-borne diseases have emerged as worldwide public health threats due to globalization and transfer of goods, along with travel and immigration (Klotz *et al*. 2010). Concerns have been raised regarding the possibility of the establishment of local transmission of Chagas disease within Australia due to the increasing number of migrants infected with *T. cruzi* from South America (Thompson. 2013). The likelihood that this exotic parasite can succeed and become established beyond the limits of their native geographical ranges might be enhanced by the presence of wildlife reservoirs that can maintain the infection, by the presence of a suitable and competent insect vector, and by the introduction of the parasites in humans or imported zoo animals. An early study showed that under experimental conditions the Australian marsupial *Trichosurus vulpecula* is susceptible to *T. cruzi* infections - *T. cruzi* was able to trigger a strong immune response in this marsupial similar to the one seen in woylies infected with *T. copemani* G2 (Backhouse *et al*. 1951, Botero *et al*. 2013). This study showed that other Australian marsupial species are susceptible to infections with different *Trypanosoma* species; thus, the possibility exists that these marsupial species could be susceptible to *T. cruzi* infections as well. There is also the possibility that the vector of *T. copemani* or any other Australian trypanosome could transmit *T. cruzi* as well. Ticks were suggested to be involved in the transmission of *T. copemani* within Australia (Austen *et al*. 2011) and were also shown to acquire the infection with *T. cruzi* when

feeding on infected dogs in South America (Dias *et al*. 2005). Moreover, an early study showed the presence of the hematophagous "kissing bug", *Triatoma leopoldi* (*Pristhesancus plagipennis*), in Cape York Peninsula in Queensland, which could be a suitable vector for *T. cruzi* like many other triatomines in Latin America (Monteith. 1974, Klotz *et al*. 2010). More recently, a new triatomine belonging to an undescribed species (*Triatoma* sp.) was described near Jabiru in the northern territory (http://www.discoverlife.org/mp/20l?id=UCR_ENT00046634). Anecdotal evidence also showed the presence of *T. leopoldi* in a house backyard in Queensland (https://www.youtube.com/watch?v=zn1e0Oma0GY), but further studies are needed to taxonomically determine if it is in fact the hematophagous bug *Triatoma leopoldi* (*Pristhesancus plagipennis*). Finally, zoo imported animals and immigrants from Latin America infected with the parasite could be the source of *T. cruzi* within Australia. Although, housing conditions within Australia are not favorable to bug colonization and the chance of human infections, in the case of *T. cruzi* introductions, is very low, Australian wildlife may be potentially naïve to *T. cruzi* infections and could be seriously affected.

The likelihood of establishment and spread of *T. cruzi* within Australia is small, but this risk should always be considered, especially in these days where the number of immigrants from Latin America and human encroachment on wildlife habitats is increasing. The understanding of the potential capacity of Australian marsupials and insects to act as reservoirs and amplifiers of emerging and exotic parasitic diseases merits further investigations.

8.3.6 Trypanosoma infections and the woylie decline

Little attention has been paid to the role of infectious agents on wildlife conservation, despite the fact of increasing evidence that they can impact significantly on local populations by driving temporary or permanent declines and extinctions (Berger *et al*. 1998, Skerratt *et al*. 2007, Ramsey *et al*. 2010). Although, wildlife hosts are thought to "have learnt to live in harmony" with trypanosomes (Legey *et al*. 1999), under certain circumstances such as stress or concurrent infections trypanosomes can become pathogenic, and therefore a significant threat to wildlife (Doherty *et al*. 1993, Seifi. 1995, Brown *et al*. 2000, Wyatt *et al*. 2008, McInnes *et al*. 2011). Trypanosomes were reported for the first time infecting the woylie in 2008 (Smith *et al*. 2008). However, this is the first study to investigate whether Australian trypanosomes can cause deleterious effects in the woylie, and consequently contribute to its population decline. The remarkably high prevalence of infection with *T. copemani* in woylies from the declining population combined with the capacity of *T. copemani* G2 to infect host cells and to cause pathology in tissues suggest that infections with this parasite may be playing an important role in the decline. Judging by the large genetic distances between all three different *Trypanosoma* species, it was remarkable than a single woylie and a single tissue could be co-infected with all species at the same time (see chapter 3). This, added to the fact that no other marsupial species examined was found co-infected with all the different species of trypanosomes, suggests that mixed infections may be contributing to the decline. It is possible that the immune response generated by a *Trypanosoma* species may act antagonistically with those elicited by another species, impairing (immuno-suppressing) simultaneous control of infections. However, further studies are needed to examine the dynamics of these co-infections and their effect on woylie host immunity.

The host immune response can also be down-regulated due to stressors such as predators, habitat lost, climate change and/or human activity (Braun *et al*. 2002, Villa *et al*. 2008), and this may also account for the increase in susceptibility of woylies to different *Trypanosoma* species. Stress may also be a factor exacerbating *Trypanosoma* infections and reducing the fitness of woylies thereby making them more vulnerable to predation (Hing *et al*. 2014, Thompson *et al*. 2014). Many pathogens, which have little or no effect on healthy animals, may cause disease in individuals that are stressed, and may have dramatic and devastating effects on naive populations. However, the fact that other small sympatric marsupials such as the common brush-tailed possum, and the quenda, that may be experiencing the same stress pressures were found infected with single infections with trypanosomes suggests that other factors might be contributing in a greater part to the susceptibility of the woylie.

It has been shown that genetically homogenous host populations are more vulnerable to pathogens and parasites than genetically diverse populations (King *et al*. 2012). Interestingly, the woylie has suffered two decline events since 1970, and has been subject to numerous translocations that have resulted in fragmented populations with reduced genetic variability (Pacioni *et al*. 2010). The low genetic diversity within the species may have resulted in an impaired or defective immune system that is unable to control infections with trypanosomes; such is the case with the Tasmanian devil and facial tumour disease (Morris *et al*. 2013). A defective immune system in the woylie may also explain the persistence of *Trypanosoma* infections in the woylie for long periods of time (Thompson *et al*. 2014), and may be facilitating the spread of trypanosomes through the woylie population. Future studies are needed to demonstrate this and to evaluate if the bottlenecks that the woylie has suffered since its first decline

(Wayne. 2008, Pacioni. 2010) has reduced its immunocompetence. Studies on the loss of diversity of the major histocompatibility complex proteins (MHC), which are critical in immune recognition of pathogens, will be a good start.

Understanding the causes of the decline is pivotal to making the best decisions in order to manage the woylie species recovery in the short to long term. The causes of the woylie decline may be common between other species that have undergone similar past and recent declines. Thus, the implications of *Trypanosoma* infections on the health of other endangered and threatened Australian native marsupials needs to be further investigated.

The examination of museum woylie specimens prior to the decline may provide important insights into understanding the infectious disease-related causes of the decline. Comparing woylie samples prior to and post decline may not only provide information about the different species of trypanosomes that were cohabiting the woylie, but it may also provide significant information about the loss of genetic diversity and implications of this on the decline. Comparing the genotypes of trypanosomes present in the woylie prior to and post decline may reveal if any introduction of new genotypes preceded the decline. Furthermore, it can be also applied to parasites such as *Toxoplasma* and *Theileria* that are known to infect the woylie.

Investigations into the woylie decline have shown a spatio-temporal pattern in its progression, with a front to the decline moving at an average of 4 km per year throughout the Upper Warren region (Wayne *et al*. 2013). Moreover, studies on the prevalence of *Trypanosoma* infections have shown a considerable increase since its first

report in 2008 (Smith *et al*. 2008, Botero *et al*. 2013, Thompson *et al*. 2014). Little is known about the mechanisms of transmission and vectors that may have facilitated the rapid spread of *Trypanosoma* infections throughout the woylie population (Figure 1) (Hamilton *et al*. 2005, Austen *et al*. 2011). The large genetic distances between all three *Trypanosoma* species described in this study and the greater prevalence of infection of *T. copemani* (97%) in the Upper Warren region (declining population) and *T. vegrandis* (89%) in the Karakamia Sanctuary (stable population) support the idea that a different arthropod vector might be involved in the transmission of each *Trypanosoma* species. The vector of *T. vegrandis* might be more prevalent in Karakamia than in the Upper Warren region, while the vector of *T. copemani* might be more prevalent in the Upper warren region than in Karakamia Sanctuary. However, judging by the high prevalence and rapid spread of *Trypanosoma* infections through the woylie population, it may be possible that mechanical or direct transmission between hosts (insect biting, oral, or sexual) may occur. Oral transmission with *T. cruzi* has been experimentally demonstrated when infected *Rhodnius prolixus* were fed to raccoons (Roellig *et al*. 2009). Furthermore, it was shown that lice of the suborder Anoplura (*Pedicinus obtusus*) are capable of disseminating *T. cruzi* infections among captive-reared baboons (*Papio hamadryas*), either by contamination of mucosal surfaces with its faeces, or by the oral route (Arganaraz *et al*. 2001).

Although this study found that some anti-trypanosomal drugs exhibited high efficacy against *T. copemani in vitro*, the possibility that these drugs can be administrated to wildlife is rather remote at this point. It will be necessary to test these drugs *in vivo* in captive woylies, before they can be safely used in wild populations. At this stage, finding the vector and investigating if others ways of transmission are involved in the

dispersion of trypanosomes is of most importance. Understanding the mechanisms of transmission of Australian trypanosomes will facilitate the development of strategies to control and limit the rate of transmission and spread of trypanosomes within Australian wildlife.

8.4 References

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Appendix I

International Journal for Parasitology: Parasites and Wildlife 2 (2013) 77–89

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Invited Article

Trypanosomes genetic diversity, polyparasitism and the population decline of the critically endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*)

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ARTICLE INFO

Article history:
Received 28 January 2013
Revised 13 March 2013
Accepted 14 March 2013

Keywords:
Australian trypanosomes
Marsupials
Tissue tropism
Intracellular
Pathogenicity
Population decline

ABSTRACT

While much is known of the impact of trypanosomes on human and livestock health, trypanosomes in wildlife, although ubiquitous, have largely been considered to be non-pathogenic. We describe the genetic diversity, tissue tropism and potential pathogenicity of trypanosomes naturally infecting Western Australian marsupials. Blood samples collected from 554 live-animals and 250 tissue samples extracted from 50 carcasses of sick-euthanized or road-killed animals, belonging to 10 species of marsupials, were screened for the presence of trypanosomes using a PCR of the 18S rDNA gene. PCR results revealed a rate of infection of 67% in blood and 60% in tissues. Inferred phylogenetic trees using 18S rDNA and glycosomal glyceraldehyde phosphate dehydrogenase (*gGAPDH*) sequences showed the presence of eight genotypes that clustered into three clades: a clade including *Trypanosoma copemani*, a new clade closely related to *Trypanosoma gilletti*, and a clade including *Trypanosoma* H25 from an Australian kangaroo. Trypanosome infections were compared in a declining and in a stable population of the endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*). This marsupial showed high rates of infection with Clade A genotypes (96%) in the declining population, whereas in the stable population, Clade B genotypes were predominant (89%). Mixed infections were common in woylies from the declining but not from the stable population. Histopathological findings associated with either mixed or single infections involving Clade A genotypes, showed a strong inflammatory process and tissue degeneration predominantly in heart, oesophagus and tongue. Trypanosomes were successfully grown in culture and for the first time we demonstrate that a genotype within Clade A has the capacity to not only colonize different tissues in the host but also to invade cells *in vitro*. These results provide evidence for the potential role of trypanosomes in the decline of a formerly abundant marsupial that is now critically endangered.

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Introduction

Trypanosomes are flagellated blood parasites that are capable of infecting virtually all classes of vertebrates. They range from non-pathogenic species to those that are highly pathogenic and are the causative agents of many diseases of medical and veterinary importance, including Chagas disease in South America and sleeping sickness and Nagana in Africa (Hoare, 1972). Some trypanosomes, normally considered as non-pathogenic, are capable of inducing detrimental effects in the host, particularly when the parasite encounters new or naïve host species following their introduction into a new habitat (Maraghi and Molyneux, 1989) or when an infected host is exposed to additional or increased levels of stress (Brown et al., 2000; Wyatt et al., 2008). This may have been the case with the introduction of *Trypanosoma lewisi* to immunologically naïve rodent hosts on Christmas Island, which caused a collapse in the population of the endemic rat *Rattus macleari* to the point of complete extinction (Pickering and Norris, 1996; Wyatt et al., 2008). Studies have also shown that *Trypanosoma theileri*, a non-pathogenic trypanosome of bovids that infects most cattle worldwide, may be considered potentially pathogenic in the presence of stress, gestation, poor nutritional status, and concurrent infections (Hussain et al., 1985; Doherty et al., 1993; Seifi, 1995).

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http://dx.doi.org/10.1016/j.ijppaw.2013.03.001

Within Australia, numerous trypanosome species and genotypes have been described naturally infecting a wide range of native marsupials. These include *Trypanosoma thylacis* in northern brown bandicoots (*Isoodon macrourus*) (Mackerras, 1959), *Trypanosoma irwini* and *Trypanosoma gilletti* in koalas (*Phascolarctos cinereus*) (McInnes et al., 2009, 2011a), *Trypanosoma binneyi* in platypus (*Ornithorhynchus anatinus*) (Noyes et al., 1999) and *Trypanosoma copemani* in quokkas (*Setonix brachyurus*), Gilbert's potoroo (*Potorous gilbertii*) (Austen et al., 2009) and koalas (McInnes et al., 2011a,b). Different genotypes are also seen in the woylie (*Bettongia penicillata*), common wombat (*Vombatus ursinus*), eastern grey kangaroo (*Macropus giganteus*), swamp wallaby (*Wallabia bicolor*) brush tailed possum (*Trichosurus vulpecula*), chuditch (*Dasyurus geoffroii*), golden bandicoot (*Isoodon auratus*), southern brown bandicoot (*Isoodon obesulus*) and burrowing bettong (*Bettongia lesueur*) (Bettiol et al., 1998; Noyes et al., 1999; Hamilton et al., 2005; Thompson et al., 2008; Smith et al., 2009; Paparini et al., 2011). Phylogenetic analysis has shown that most of these trypanosomes fall into several distinct clades. Some of them are closely related to trypanosomes from outside Australia, but the majority cluster with species/genotypes within Australia (Stevens et al., 2001; Hamilton et al., 2005; Thompson et al., 2008; Smith et al., 2009; Austen et al., 2009; McInnes et al., 2011a; Paparini et al., 2011). Despite the identification of this large list of trypanosomes and their marsupial hosts, there are many unanswered questions related to host–parasite interactions and their pathogenic potential during single and mixed natural infections.

A commonly infected Australian native marsupial is the woylie (Thompson et al., 2008; Smith et al., 2009), which occupied most of the southern half of the Australian mainland before European settlement in 1788. However by the 1970s, woylies were listed as critically endangered due to a drastic reduction in abundance from habitat destruction and introduced predators (Start et al., 1995; Orell, 2004; de Tores et al., 2008). The geographical distribution of the woylie became confined to three locations in Western Australia (WA), the Upper Warren Region, Tutanning Nature Reserve and Dryandra Woodland (Fig. 1) (DEC, 2008; de Tores et al., 2008). Significant conservation efforts, which included predator control and captive breeding and release, led to an increase in abundance from the mid 1970s into the early 2000s. As a consequence of these efforts, woylies were removed from the endangered species list in 1996 (Start et al., 1998). However, since 1999, remaining populations have undergone a dramatic 90% reduction in abundance despite no apparent increase in the number or type of predators in the region and no apparent decrease in natural resources (Wayne et al., in press a,b). As a consequence of these population declines, woylies were included once again on the endangered species list (Wayne et al., 2008; Groom, 2010). Karakamia wildlife sanctuary, a predator-proof fenced reserve located 50 km north-east of Perth, is the only substantial translocated population of woylies that has remained stable in Western Australia. Disease, possibly caused by *Trypanosoma* parasites shown to be at high prevalence levels, has been under consideration as an important factor associated with the decline (Thompson et al., 2008).

Considering the pathogenic potential of trypanosomes when encountering new host species or when an infected host is exposed to increased levels of stress, it is becoming increasingly important to establish their presence, rate of infection, genetic diversity and phylogenetic status within Australian wildlife. To help clarify these issues, this study aims to: (i) determine the genetic variability and phylogenetic relationships of trypanosomes circulating in Western Australia marsupials; (ii) investigate the life cycle of trypanosomes in the marsupial host; (iii) characterize trypanosome behavior in axenic culture; and (iv) evaluate the pathogenicity of trypanosomes and their influence in the decline of the woylie. Together these data will help clarify the impact of trypanosomes on the health of native Australian marsupials.

Materials and methods

Sample collection

Tissue and blood samples were collected from 10 species of marsupials at different locations throughout Western Australia (WA) as part of the WA Department of Environment and Conservation (DEC) fauna research and monitoring program (Fig. 1, Table 1). A total of 554 heparinized peripheral blood samples were collected from trapped and released marsupials during ongoing trapping sessions by DEC. 237 of these samples were collected from woylies at the stable population in Karakamia Sanctuary and 257 from woylies at the declining population in the Upper Warren Region. Tissue samples were collected from sick-euthanized animals that were presented to Perth Zoo Veterinary Department for treatment and from dead (accidently killed on roads) animals sent to Murdoch University for necropsy. Sick animals were euthanized due to very poor body condition, marked lethargy and poor prognosis for return to the wild. A total of 250 tissue samples were collected from 50 carcasses and at least two of the following tissues were collected from each animal: spleen, liver, lung, heart, kidney, brain, oesophagus, tongue, skeletal muscle and bone marrow. Wildlife sampling was carried out under Murdoch University animal ethics approval permit numbers NS1182-06, W2172-08 and W2350-10, and DEC animal ethics approval permit number DECAEC/52/2009. All tissue samples were extensively washed with phosphate buffered saline (PBS) and stored in 100% ethanol and 10% formalin for DNA isolation and histopathological analysis respectively.

Isolation, growth behavior and morphology of trypanosomes

Cultures were established by inoculation of approximately 50 μl of peripheral blood in biphasic media containing Brain-Heart Infusion (BHI) medium with agar and 10% defibrinated horse blood as a solid phase, and either RPMI or HMI9 medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) and 50 μg/ml Penicillin–Streptomycin as a liquid phase. All isolates were expanded in liquid media until they reached a density of approximately 1×10^6 parasites/ml and were subsequently maintained with successive passages every week at 27 °C in the dark. Thin blood smears taken from naturally infected marsupials and smears of logarithmic and stationary phase cultures were fixed in methanol and stained with the commercial Romanowsky-type stain 'Diff-Quik' for examination by light microscopy. For scanning electron microscopy (SEM), culture forms were fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS: cell culture media (pH 7.2), before being mounted on poly-L-lysine coated coverslips, progressively dehydrated through a series of ethanols and critical point dried as previously described (Edwards et al., 2011). Coverslips were mounted on stubs with adhesive carbon, coated with 2 nm Pt and imaged at 3 kV using the in-lens secondary electron detector on a Zeiss 55VP field emission SEM.

Cell infection

Metacyclic trypomastigotes (1.4×10^4/well) from one week old cultures growing in liquid media were used to infect monolayers of L6 (skeletal muscle) and Vero (kidney epithelial) cells grown to 50% confluency in RPMI media supplemented with 10% FCS, on 16 well glass coverslips. In parallel, cells were infected with *Trypanosoma cruzi* Tulahuen strain for comparison. All cells were maintained at 37 °C and 5% CO_2. Two days post-infection, cells

Fig. 1. Geographical origin of the different species of marsupials trapped in this study.

Table 1
Overall prevalence of *Trypanosoma* infection in blood and tissues of different species of Western Australian marsupials.

Marsupial species	Blood samples (%, 95% CI)	Carcasses (%, 95%CI)	Tissue samples (%, 95% CI)
Woylie (*Bettongia penicillata*)	335/494 (68, 63–71)	18/27 (67, 46–81)	67/154 (43, 35–51)
Quenda (*Isoodon obesulus*)	0/2	2/2	8/11
Quokka (*Setonix brachyurus*)	–	1/3	2/7
Tammar wallaby (*Macropus eugenii*)	–	1/3	3/7
Banded hare wallaby (*Lagostrophus fasciatus*)	1/1	–	–
Boodie (*Bettongia lesueur*)	1/7	0/1	0/3
Chuditch (*Dasyurus geoffroii*)	1/2	–	–
Common brush tailed possum (*Trichosurus vulpecula*)	3/7	–	–
Western grey kangaroo (*Macropus fuliginosus*)	29/41	4/9	9/38
Quoll (*Dasyurus maculatus*)	–	4/4	17/30
Total	370/554 (67, 63–71)	30/50 (60, 48–75)	106/250 (42, 36–48)

The rate of infection is given by: number of *Trypanosoma*-positive samples or carcasses/total number of samples or carcasses. 95% CI (95% confidence interval).

were washed three times with PBS to remove extracellular parasites, the coverslips were then removed, air-dried and stained with Romanowsky 'Diff-Quik' staining system for optical microscopy. For SEM, glass cover slips with attached trypanosome-infected cells were removed from the culture plates and processed and imaged as described above. For transmission electron microscopy (TEM), trypanosome-infected cells were washed three times with PBS, trypsinized with 0.25% trypsin/EDTA (GIBCO) for a few minutes at 37 °C to detach the cells from the culture plates and resuspended in fresh media. This cell suspension was then fixed in the glutaraldehyde:culture media mixture. All subsequent processing was performed in a PELCO Biowave microwave, where samples were post-fixed in 1% OsO_4 in PBS followed by progressive dehydration in ethanol/acetone, before being infiltrated and embedded in epoxy resin Procure-Araldite. Sections 120 nm thick were cut on a diamond knife and mounted on copper grids. Digital images were collected from unstained sections at 120 kV on a JEOL 2100 TEM fitted with a Gatan ORIUS1000 camera.

PCR amplification and sequencing of 18S rDNA and gGAPDH loci

Genomic DNA from blood, tissue and cultured trypanosomes was obtained using the QIAamp blood and tissue DNA MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A nested PCR that targets a variable region of the 18S rDNA locus using generic primers that have the potential to recognize different species of trypanosomatids was performed as described previously (Maslov et al., 1996; McInnes et al., 2011a). Positive samples with 18S rDNA PCR were screened with a second PCR of the *gGAPDH* locus using modified heminested reactions (McInnes et al., 2011a). To investigate the genetic diversity of trypanosomes infecting Western Australian marsupials, 200 positive PCR products from blood and tissue and 28 PCR products from trypanosomes grown in culture were chosen, purified using Agencourt AMPure PCR Purification system (manufacturer's instructions) and sequenced (18S rDNA: ~1.5 kb and *gGAPDH*: ~810 bp) using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Bio-systems, California, USA) on an Applied Bio-system 3730 DNA Analyzer. Chromatograms, derived from complete sequences, were used for contiguous assembly using the DNA sequence analysis program Sequencher version 5.0.1.

DNA sequence alignments and phylogenetic inferences

18S rDNA and g*GAPDH* sequences obtained from blood, tissue and cultured trypanosomes, were aligned using MUSCLE (Edgar, 2004) and then manually refined using BioEdit 7.0.9.0 (Hall, 1999). Three different alignments were created for phylogenetic inference. First, 1410 bp sequences of 18S rDNA from trypanosomes obtained in the current study were aligned with 31 *Trypanosoma* spp. sequences representing all known trypanosome clades, and five other trypanosomatid sequences for use as outgroups obtained from GenBank. Secondly, truncated (786 bp) sequences of 18S rDNA were aligned with those previously published from different Western Australian marsupials (Thompson et al., 2008; Smith et al., 2009; Austen et al., 2009). Finally, 810 bp g*GAPDH* sequences obtained in the current study were aligned with 26 *Trypanosoma* spp. sequences representing all major trypanosome clades, and five other trypanosomatid sequences for use as out-

groups. All *Trypanosoma* spp. and outgroup sequences obtained from GenBank are shown in Table 2. Phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian methods, implemented using MEGA 5 (Tamura et al., 2011) and Mr Bayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Genetic distances were also calculated using the Kimura 2 parameter model within MEGA 5. Nodal support was estimated with 10,000 bootstrap replicates. JModelTest 2.1.1 was used to find the most appropriate nucleotide substitution model for ML and Bayesian analyses (Posada, 2008). The models of nucleotide substitution chosen were: TIM3ef+I+G (equal-frequencies transition model plus gamma) for the first alignment and GTR+I+G (general time reversible gamma proportion of invariant sites) for the other two alignments. The Markov chain Monte Carlo was run for 10,000,000 generations, until the mean standard deviation of split frequencies was lower than 0.01, and the trees were sampled every 100th generation. The first 2500 trees were discarded as burn-in.

Trypanosome clade-specific PCR

Trypanosome clade-specific primers were constructed based on the aggregation of the different genotypes in the phylogenetic tree obtained from the 1410 bp 18S rDNA sequences (see Results). Specific primers that amplify *T. copemani* and could recognize genotype 1 and genotype 2 (both in Clade A) were used as described previously (McInnes et al., 2011a). Two sets of specific primers that amplify the other major genotype groups, genotypes 3–7 (Clade B) and G8 (Clade C) were constructed as shown in Table 3. PCR reactions for all blood and tissue samples were performed in a total volume of 25 µl containing 0.2 U Taq DNA Polymerase, 200 M of dNTPs, 0.8 µM of each primer and 1 µl of DNA template. Amplification was performed in a PT100 thermocycler (MJ Research) and consisted of a pre-PCR step at 94 °C for 5 min, 50 °C for 2 min and primer extension at 72 °C for 4 min followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature described in Table 3 for each clade, 50 s at 72 °C and a final extension step at 72 °C for 7 min. PCR products were run on a 1.5% agarose gel stained with SYBR safe (Invitrogen, USA), and visualized with a dark reader trans-illuminator (Clare Chemical Research, USA). Prevalence of infection with trypanosomes was expressed as the percentage of samples found positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000).

Histopathological analysis

PCR positive tissues were paraffin embedded and 3 µm-thick sections were cut and stained with hematoxylin and eosin (H&E). Each section was examined microscopically for inflammatory lesions and the presence of trypomastigotes in blood vessels and/or capillaries and amastigotes in tissue cells. Sections for histopathological analysis were only collected from tissue of woylies, quokkas, quendas and Western grey kangaroos.

Results

General rate of infection in blood and tissue revealed by PCR

Blood samples from 554 trapped and released marsupials and 250 tissue samples extracted from 50 carcasses of dead or euthanized marsupials were screened for the presence of trypanosomes using generic primers for 18S rDNA. At least one individual of each marsupial species examined was positive for *Trypanosoma* infection either in blood or tissue. The rate of infection over all host species was 67% in blood of trapped and released animals and 60% in carcasses of dead animals, where at least one tissue sample was positive (Table 1).

Table 2
GenBank accession number of the sequences used in the phylogenetic analysis.

Isolates	18S rDNA Accession number	*gGAPDH* Accession number
T. rotatorium	AJ009161	AJ620256
AAT (Currawong)	AJ620557	
AAI	AJ620559	–
KG1 (Tick)	–	FJ649492
T. mega	AJ009157	AJ620253
T. binneyi	AJ132351	AJ620266
T. granulosum	AJ620551	–
T. avium Rook	U39578	–
T. theileri	AJ009164	AJ620282
T. cyclops	AJ131958	
ABF (Wallaby)	AJ620564	AJ620278
H25 (Kangaroo)	AJ009168	AJ620276
H26	AJ009169	–
T. dionisii	AJ009151	FN599054
T. cruzi marinkellei	AJ009150	AJ620270
T. cruzi COLOMBIANA	AF239980	–
T. cruzi SLU31 clone2	AY785586	–
T. cruzi VINCH89	AJ009149	AJ620269
T. rangeli	AJ009160	AF053742
T. vespertilionis	AJ009166	AJ620283
T. conorhini	AJ012411	AJ620267
T. pestanai	AJ009159	AJ620275
AAP	AJ620558	AJ620277
T. lewisi	AJ009156	AJ620272
T. microti	AJ009158	AJ620273
T. gilletti Lanie	GU966589	GU966587
T. copemani Charlton (Koala)	GU966588	GU966585
T. copemani Mika (Koala)	–	GU966585
T. copemani Harrison (Koala)	–	GU966586
T. irwini	FJ649479	FJ649485
T. bennetti	AJ223562	FJ649486
T. copemani Q3 (Quokka)	EU571232	–
T. copemani Q10 (Quokka)	EU571234	–
T. copemani GP94 (Gilbert's potoroo)	EU571231	–
T. copemani GP63 (Gilbert's potoroo)	EU571233	–
TRY1 (Woylie)	EU518939	–
TRY2 (Woylie)	EU518940	–
WYA1 (Woylie)	FJ823116	–
WYA2 (Woylie)	FJ823121	–
CHA1 (Chuditch)	FJ823120	–
T. sp. AP2011 isolate27 clone4	JN315394	–
T. sp. AP2011 isolate4 clone6	JN315392	–
T. sp AP2011 isolate28 clone11	JN315387	–
T. sp. AP2011 isolate 15	–	JN315395
T. sp. AP2011 isolate 17	–	JN315396
Leptomonas sp.	AF153043	AF339451
Phytomonas serpens	U39577	EU084892
H. muscarum	L18872	DQ092548
H. samuelpessoai	U01016	AF047494
H. megaseliae	U01014	DQ092547

DNA sequences and phylogenetic analysis

Eight different genotypes (G1 to G8) were found at the 18S rDNA locus among the 200 blood and tissue samples chosen for sequencing (Table 4). The sequences obtained from trypanosomes grown in culture revealed all of them were G1 and G2. Very similar phylogenetic relationships were found using both Bayesian and ML analyses between these eight genotypes and the 31 trypanosome sequences obtained from GenBank; we show only the results from the Bayesian analysis here. The eight genotypes found in our study were grouped into three distinct clades (Fig. 2A). The first two genotypes (G1 and G2) clustered within Clade A; G1 showed a 99% and 98% similarity to *T. copemani* previously described from

Table 3
Clade-specific PCR conditions and primer sequences.

PCR	Primer name	Primer sequence	$MgCl_2$ concentration	Annealing temperature	Band size
Clade C External	H25EF	GCCGACAGTGCATTTTGT	1.0 mM	60	~750 bp
	H25ER	GAGCGAGATGAACTCGACC			
Clade C Internal	H25IF	TTTGAGGCGCAATGGTTTAG	1.0 mM	60	~400 bp
	H25IR	CGAGTTGAGGGAAGGTGGC			
Clade B External	TVEF	GGGGTCCTTTTATTTTATTTG	1.5 mM	58	~750 bp
	TVER	TAATTTATTGGCCAGACAAA			
Clade B Internal	TVIF	GACCAAAAACGTGCACGTG	1.0 mM	58	~350 bp
	TVIR	AAATCGTCTCCGCTTTAAC			

Table 4
GenBank accession number of the new reported sequences and origin of the isolates.

GenBank Accession number		*Trypanosoma* Genotype	*Trypanosoma* Clade	Hosts
18S rDNA	*gGAPDH*			
KC753530	KC812982	Genotype 1 (G1)	Clade A	Woylie, common brush-tailed possum
KC753531	KC812983	Genotype 2 (G2)	Clade A	Woylie, quokka, quoll, quenda
KC753533	KC812984	Genotype 3 (G3)	Clade B	Woylie, Western grey kangaroo
KC753532	KC812985	Genotype 4 (G4)	Clade B	Woylie, quenda
KC753534	KC812986	Genotype 5 (G5)	Clade B	Woylie, tammar wallaby
KC753535	-	Genotype 6 (G6)	Clade B	Woylie, chuditch
KC753536	KC812987	Genotype 7 (G7)	Clade B	Woylie
KC753537	KC812988	Genotype 8 (G8)	Clade C	Woylie, banded hare wallaby, boodie

a wombat and koalas (Noyes et al., 1999; McInnes et al., 2011a), and G2 was closely related to G1 but differed in 13 nucleotides among the 1410 bp of sequence. Five genotypes (G3 to G7) clustered in Clade B together with *T.* sp AP2011 isolate 27, *T.* sp. AP2011 isolate 28 and *T.* sp. AP2011 isolate 4 (Paparini et al., 2011). In all analyses, the closest relative of this clade among previously described trypanosome species was *T. gilletti* from koalas, although the nucleotide distance between *T. gilletti* and the genotypes within this clade was considerably greater than distances between many closely related *Trypanosoma* species (Table 5). The last genotype found (G8) presented 99% similarity with a trypanosome isolated from an Australian kangaroo – *Trypanosoma* H25 (Noyes et al., 1999). Genotypes within this clade (Clade C) were more closely related to trypanosomes from outside Australia such as *T. cruzi* and *Trypanosoma rangeli* from South America and were previously positioned in a monophyletic assemblage designated as "*T. cruzi* clade" (Hamilton et al., 2012). Phylogenetic analysis of the eight new genotypes found in this study and previously published, truncated 18S rDNA sequences from trypanosomes isolated from Western Australian marsupials showed close relationships between them. Trypanosome isolates from a chuditch (CHA1) and woylies (TRY1, TRY2, WYA1, WYA2), clustered within Clade B, while *T. copemani* isolates from quokkas (Q3 and Q10) and Gilbert's potoroos (GP63 and GP94) were more closely related to G1 than to G2 (Fig. 2B).

Additional support for the phylogenetic positioning of the new sequences was provided by the phylogenies derived from the g*GAPDH* sequences (Fig. 3), which showed the same topology as the 18S rDNA derived tree, although only seven genotypes were included due to the lack of PCR amplification of G6.

Trypanosome clade-specific PCR

To confirm reliability of trypanosome clade-specific primers and PCRs, DNA sequencing was carried out from 16 blood samples infected with genotypes representative of the three different clades. These sequences showed a 100% similarity with the full 18S rDNA sequences, and confirmed the high specificity of the clade-specific primers and PCRs.

Out of the 370 blood samples positive for trypanosome infection, 53% (95% CI = 49–57) were positive for Clade A, 32% (95% CI = 27–37) for Clade B, 2% (95% CI = 1–3) for Clade C and 13% (95% CI = 10–17) were mixed infections with two or more genotypes representative of different clades. From the 106 tissues infected, 47% (95% CI = 38–57) were positive for Clade A, 29% (95% CI = 21–39) for Clade B, 2% (95% CI = 0.3–7) for Clade C and 22% (95% CI = 14–31) for mixed infections. Although PCR using clade-specific primers showed the presence of mixed infections in some of the samples, sequencing of the same samples using the 18S rDNA trypanosome generic primers revealed infections with only one genotype.

Parasite genotypes were not host-specific, with most being found in at least two host species (Table 4). Whereas single infections were found in blood and different tissues of all marsupials examined, mixed infections were only present in blood and tissues of woylies. This host species presented a total of 13% (95% CI = 10–17) and 1% (95% CI = 0.2–3) of dual infections with genotypes belonging to Clade A–Clade B and Clade B–Clade C respectively. Two percent of the samples (95% CI = 0.8–4) presented triple infections with genotypes from the three clades.

All trypanosome genotypes were present in peripheral blood. However, only G2 (Clade A), G3–G5 (Clade B) and G8 (Clade C) were found infecting host tissues, including tissues from woylies, quokkas, quendas, tammar wallabies, quolls and western grey kangaroos. Trypanosomes within Clade A exhibited a preferential tissue distribution for skeletal muscle, tongue, oesophagus, and heart, while trypanosomes within Clade B showed predilection for skeletal muscle, heart and lung. Table 6 shows the rate of infection among all different tissues collected, including tissues infected with more than one genotype.

Comparison of trypanosome infections between stable and declining populations of woylies

Woylies showed a rate of infection of 68% in blood of trapped and released animals and 67% in carcasses of dead and euthanized animals (Table 1). There was a significantly higher prevalence of trypanosome infection in sick and euthanized woylies compared with those found dead on roads; while only four out of 12 road kills were infected with trypanosomes, almost all (nine out of 10) euthanized animals were positive for trypanosome infection (Fisher exact test, $P = 0.01$). There was a signifi-

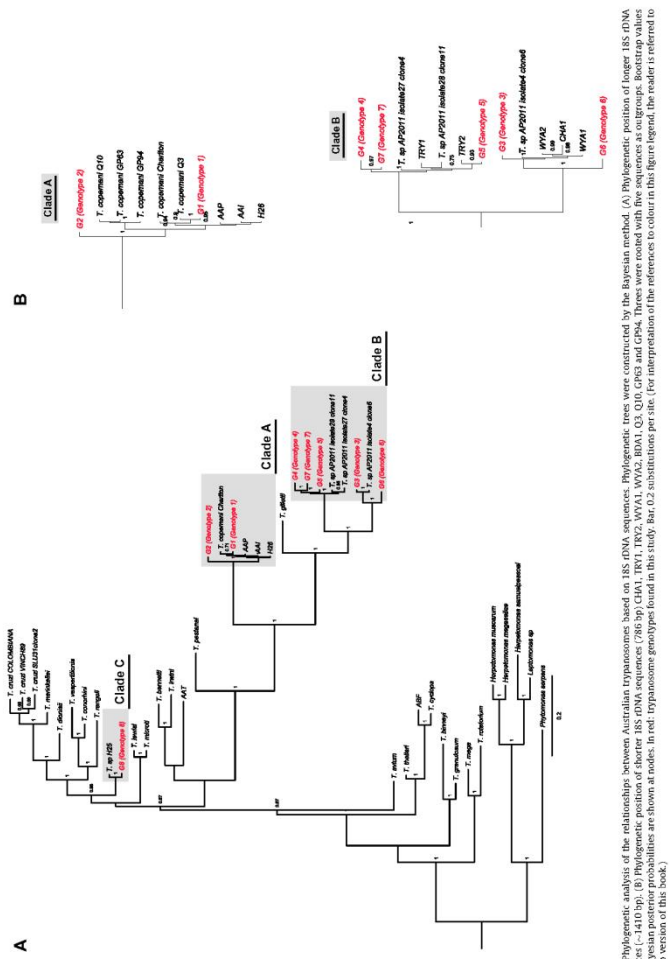

Fig. 2. Phylogenetic analysis of the relationships between Australian trypanosomes based on 18S rDNA sequences. Phylogenetic trees were constructed by the Bayesian method. (A) Phylogenetic position of longer 18S rDNA sequences (~1410 bp). (B) Phylogenetic position of shorter 18S rDNA sequences (~265 bp). CHA1, TRY1, TRY2, WYA1, WYA2, BDA1, Q3, Q10, GPK3 and GPK4. Threes were rooted with five sequences as outgroups. Bootstrap values from Bayesian posterior probabilities are shown at nodes. In red: trypanosome genotypes found in this study. Bar, 0.2 substitutions per site. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

Table 5
Genetic distances based on the 18S rDNA sequences from representative genotype sequences of each *Trypanosoma* clade found in this study; sequences from other *Trypanosoma* species are also shown. Analyses were conducted using the Kimura 2-parameter model within MEGA5. Genotype 2 (Clade A), genotype 3 (Clade B), genotype 8 (Clade C).

	Genotype 2	Genotype 3	Genotype 8	*T. gilletti*	*T. cruzi*	*T. rangeli*	*T. lewisi*
Genotype 2	–	0.154	0.132	0.120	0.152	0.157	0.139
Genotype 3	–	–	0.162	0.104	0.189	0.203	0.178
Genotype 8	–	–	–	0.137	0.052	0.048	0.052
T. gilletti	–	–	–	–	0.153	0.171	0.154
T. cruzi	–	–	–	–	–	0.087	0.069
T. rangeli	–	–	–	–	–	–	0.076
T. lewisi	–	–	–	–	–	–	–

Fig. 3. Phylogenetic relationships of the new trypanosome isolates from Western Australian marsupials based on *gGAPDH* sequences (~810 bp) using Mr Bayes. The tree was rooted with five sequences as outgroups. Bayesian posterior probabilities are shown at nodes. Bar, 0.07 substitutions per site.

cant difference in the prevalence of infection with genotypes from Clade A and Clade B in the stable and declining populations (Fisher exact test, $P = 0.0001$). Among positive samples, the genotypes most frequently found in the stable population were from Clade B, present in 89% (95% CI = 82–94) of the samples. In contrast, genotypes from Clade A were most frequently found in the declining population, present in 96% (95% CI = 93–98) of the samples. Mixed infections were also found more frequently in the declining than in the stable population (Fisher exact test, $P = 0.001$) (Fig. 4).

Table 6
Differential tissue distribution of trypanosomes within the different clades.

Trypanosoma clades	Spleen	Kidney	Liver	Lung	Heart	Tongue	Skeletal muscle	Oesophagus	Brain	Bone marrow
Clade A	10% (2–26)	17% (5–37)	25% (13–43)	21% (9–37)	37% (19–58)	40% (16–68)	41% (25–59)	38% (14–68)	0%	9% (2–41)
Clade B	7% (1–22)	12% (26–32)	8% (16–21)	16% (60–31)	18% (6–38)	13% (16–40)	47% (29–65)	15% (19–45)	0%	9% (2–41)
Clade C	3% (0.1–17)	4% (0.1–21)	3% (0.1–14)	5% (0.6–18)	0%	0%	0%	0%	0%	0%
TOTAL	20% (8–38)	33% (16–55)	37% (22–54)	42% (26–59)	56% (35–74)	53% (26–79)	88% (72–97)	54% (25–81)	0%	18% (2–52)

The rate of infection (%) in each organ is given by the number of *Trypanosoma*-positive organs/total number of organs. Between brackets: 95% confidence interval (95% CI).

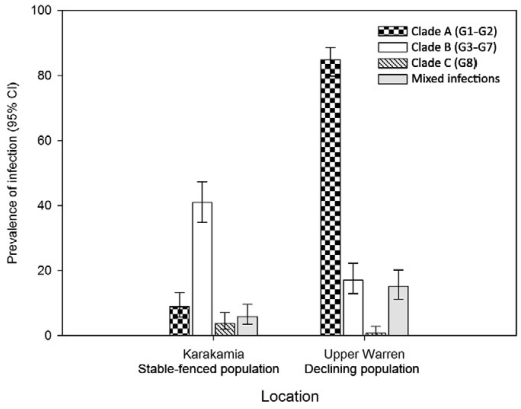

Fig. 4. Prevalence of infection with trypanosomes within the different clades in woylies from the stable and declining populations. 95% confidence intervals (95% CI).

Histopathological analysis

Gross pathology from 13 woylie carcasses that were infected with trypanosomes showed poor body and coat conditions and heavy infestation with ectoparasites (lice and ticks); most of them exhibited areas of alopecia accompanied by chronic multifocal exudative dermatitis.

Tissue sections taken from animals infected with genotypes either from Clade B or Clade C, including the woylie, Western grey kangaroo, and quenda, showed an absence of tissue lesions associated with trypanosomiasis. However, a mild inflammatory process was seen in tissues from woylies with a dual infection of genotypes from these two trypanosome clades. A moderate to marked inflammatory process was seen in tissues infected either with genotypes from Clade A or in co-infections involving trypanosomes from this clade. This process consisted, in general, predominantly of plasma cells, lymphocytes, macrophages, neutrophils and mast cells. The pathology seen in heart sections of three woylies showed a multifocal, severe and chronic pyogranulomatous myocarditis and endocarditis accompanied by muscle degeneration and necrosis (Fig. 5A–C). A multifocal, chronic, pyogranulomatous oesophagitis and glossitis, both accompanied by prominent skeletal muscle degeneration was also seen (Fig. 5D and E). Occasionally, inflammatory cells clustered around blood vessels (Fig. 5F). Trypanosomes were not observed intravascularly. However three woylies and one quenda presented structures suggestive of amastigotes in heart tissue (Fig. 6A and B). Histopathology of 10 sections taken from different tissues of two non-infected road kill woylies showed an absence of tissue lesions.

Growth behavior in culture and morphology of trypanosomes

Because woylies exhibited high levels of parasitemia and were detected harboring all different trypanosome genotypes found in this study, additional peripheral blood samples were taken and cultured in biphasic media from animals at Karakamia (stable population) and Upper Warren (declining population).

From 30 haemocultures established from blood of the stable population, no evidence of protozoa morphologically similar to trypanosomes was seen after 30 days of culture in biphasic media, either with RPMI or HMI9 media as a liquid phase. Furthermore, no trypanosomes were detected in direct peripheral blood smears from these animals, but PCR of extracted DNA from blood revealed 21 of them were infected with genotypes belonging to Clade B. In contrast, from the declining population, trypanosomes were detected in 24 of 30 haemocultures after day 8 to day 20 post-inoculation. PCR and sequencing of the 18S rDNA locus revealed all isolates were G1 and G2 (Clade A). Peripheral blood smears from the declining population showed blood forms consisted of large trypomastigotes, a small kinetoplast positioned far from the nucleus, and a well developed undulating membrane and flagellum (Fig. 7A). Dividing trypomastigote forms were never observed in blood smears.

Both genotypes in Clade A were successfully subcultured in biphasic media for up to 6 months at weekly intervals, but they differed in growth requirements when only liquid media was used. G1 showed a growth rate significantly higher in RPMI media while the growth of G2 was much more efficient in HMI9 media. Nevertheless, morphological features were almost identical between them. Eight-day-old cultures showed slender trypomastigotes that exhibited a rounded kinetoplast positioned close to the posterior end (Fig. 7B). Epimastigotes presented highly variable shapes with the kinetoplast positioned adjacent to the nucleus (Fig. 7C and D); some of the forms were spheromastigotes that were often seen to be dividing (Fig. 7E and F). Eight-day-old cultures also presented epimastigotes that were longer and thinner, most were undergoing binary division, giving rise to rosettes and transition forms of variable shape, length of body and flagellum (Fig. 8A). All forms growing in culture presented numerous acidocalcisomes randomly distributed along the whole body (Fig. 8B).

Cell infection

Trypanosome isolates G1 and G2 (Clade A) were used to infect Vero and L6 cells *in vitro*. Only G2 was able to infect cells. Amastigotes of G2 and *T. cruzi* (our positive control of infection) were observed inside cells at day 2 post-infection (Fig. 9A and B). Fig. 10A and B shows trypomastigotes invading a cell and dead cells surrounded by amastigotes and trypomastigotes that were frequently found after the second day of infection. Transmission electron microscopy of trypomastigotes of G2 confirmed the presence of acidocalcisomes and showed the flagellum at the surface of the cell containing the axoneme (AX) made of nine doublets of microtubules

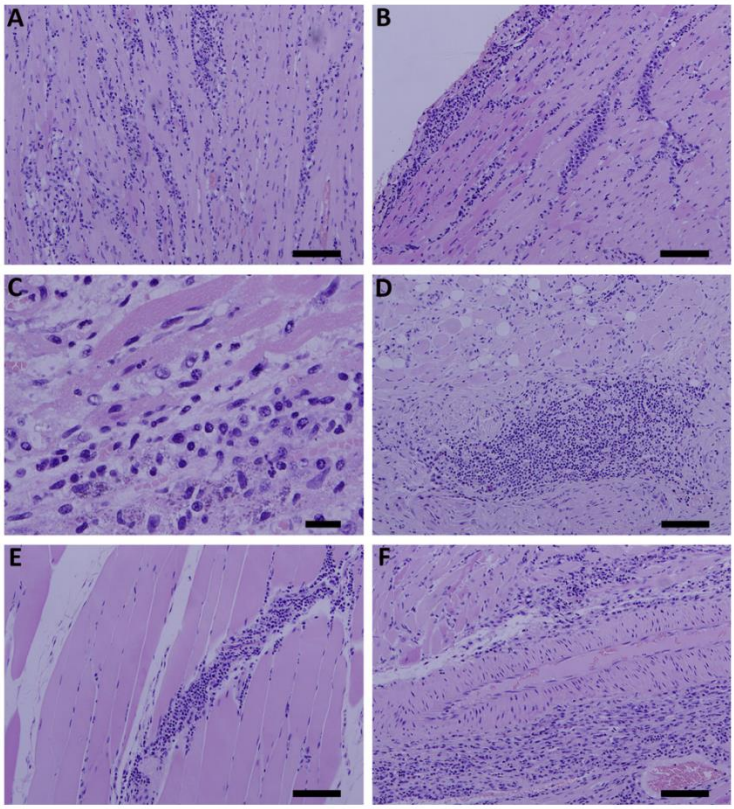

Fig. 5. Histopathology of two woylies naturally infected with G2 (Clade A) (H&E stained). (A) Multifocal, moderate to severe, chronic, pyogranulomatous myocarditis and (B) endocarditis. (C) Mineralisation of heart tissue. (D) Tongue showing multifocal, moderate, chronic, pyogranulomatous glossitis. (E) Skeletal muscle degeneration. (F) Inflammatory cells around a blood vessel. Scale bars = 20 μm.

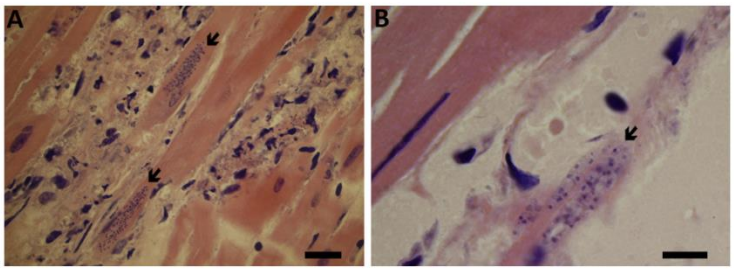

Fig. 6. Structures suggestive of amastigotes (arrows) of G2 (Clade A) in heart tissue positive by PCR (H&E stained). Scale bars = (A) 20 μm, (B) 10 μm.

surrounding a central pair as in most flagellated eukaryotes (Fig. 11A). Fig. 11B shows an amastigote inside a Vero cell.

Discussion

This study has confirmed previous findings that trypanosomes in Australian marsupials comprise a heterogeneous community, with low levels of host specificity and no evidence of restricted geographical distribution. *T. copemani* has been found in the blood of quokkas and Gilbert's potoroos from Western Australia (Austen et al., 2009), in koalas from Queensland (McInnes et al., 2011a,b), and in a wombat from Victoria (Noyes et al., 1999). *T. gilletti* and numerous closely related trypanosome genotypes have been reported in the blood of koalas from Queensland (McInnes et al., 2011a,b) and woylies from Western Australia (Thompson et al., 2008; Smith et al., 2009; Paparini et al., 2011). *Trypanosoma* H25, which was found to cluster on a phylogeny with *T. cruzi*, has been reported in the blood of a kangaroo from Victoria (Noyes et al., 1999) and in possums from Western Australia (Paparini et al., 2011). Here, we have found genotypes closely related to *T. copemani* in woylies, quendas, quolls and common brush tailed possums; a number of genotypes related to *T. gilletti* in the Western

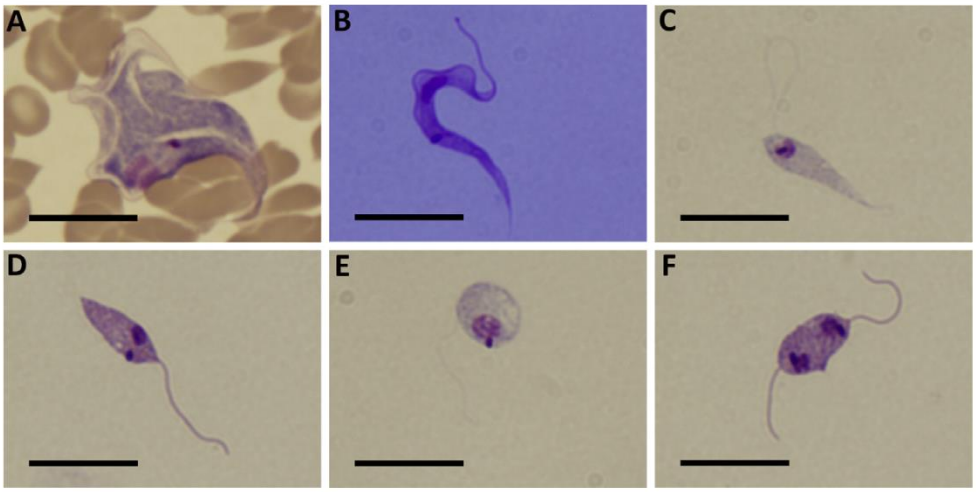

Fig. 7. Light microscopy of Diff-Quick stained blood and culture forms of G1 and G2 (Clade A) (A) Trypomastigote in blood of a woylie naturally infected; (B) slender epimastigote in culture; (C and D) shaped epimastigote in culture; (E) spheromastigote in culture; (F) spheromastigotes dividing in culture. Scale bars = 10 µm.

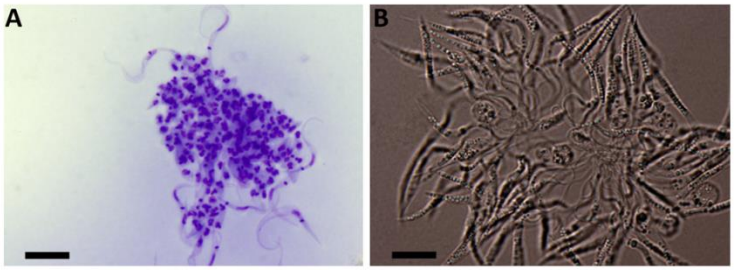

Fig. 8. Epimastigotes of G1 and G2 (Clade A) arranged in rosettes in culture. (A) Diff-Quick stained rosettes. (B) Rosettes in fresh wet preparations showing numerous intracellular acidocalcisomes. Scale bars = 10 µm.

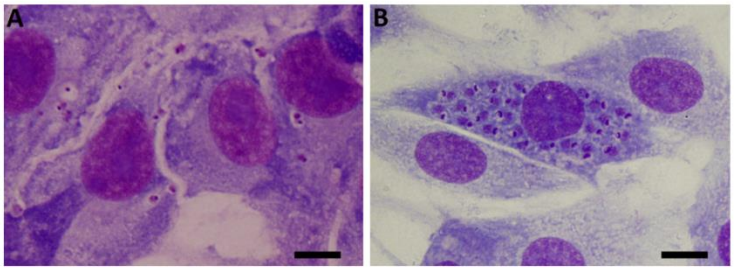

Fig. 9. Infection of Vero (A) and L6 cells (B) with G2 (Clade A) and *T. cruzi* as a positive control of infection (Diff-Quick stained). (A) Intracellular amastigotes of G2. (B) Intracellular amastigotes of *T. cruzi*. Scale bars = 10 µm.

grey kangaroos, quenda, tammar wallaby and chuditch; and a genotype closely related to *Trypanosoma* H25 in woylies, banded hare wallabies and boodies from Western Australia. Our data also demonstrate that marsupials belonging to several families in the same locality could be infected with the same trypanosome genotype. This lack of host specificity in Australian trypanosomes may have important consequences for the evolutionary and ecological interactions between trypanosomes and their marsupial hosts.

To infer phylogenetic relationships between the eight trypanosome genotypes found in this study and previously described species, we sequenced both 18S rDNA and *gGAPDH* genes. The use of partial 18S rDNA sequences alone is considered inadequate for inferring deep levels of phylogenetic relationships and additional gene sequences are needed to help resolve polytomies in the Trypanosomatidae (Hamilton et al., 2004, Hamilton and Stevens, 2011; Lymbery et al., 2011). Phylogenetic trees inferred in this study using both loci yielded almost identical topologies, and the general branching pattern was in agreement with those shown in previous analyses based on single and combined 18S rDNA and *gGAPDH* sequences (McInnes et al., 2009; Paparini et al., 2011;

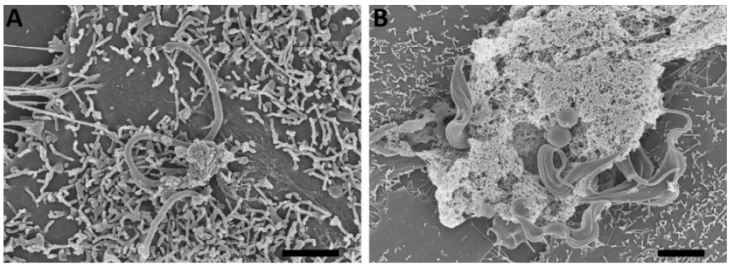

Fig. 10. Scanning electron micrograph of G2 (Clade A) grown in culture with Vero cells. (A) Trypomastigote invading a cell, with the flagella still external to the cell. (B) Dead cell(s) surrounded by amastigotes and trypomastigotes. Scale bars = 2 μm (A), 4 μm (B).

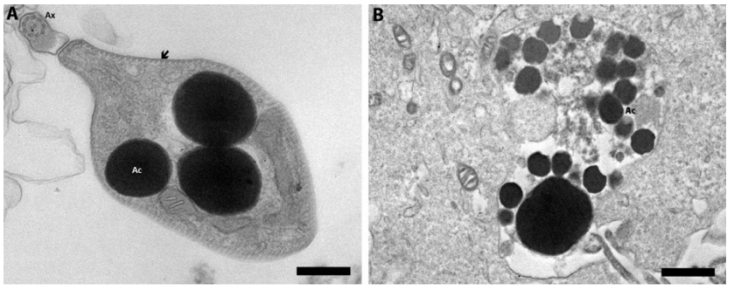

Fig. 11. Transmission electron micrograph of an epimastigote and an amastigote of G2 (Clade A). (A) Epimastigote in culture; Ax: Axoneme showing nine doublets of microtubules surrounding a central pair; Ac: Acidocalcisomes; Arrow: Subpellicular microtubules. (B) Amastigote inside a VERO cell. Scale bars = 0.5 μm (A), 1 μm (B).

Hamilton et al., 2012). Our analysis showed two of the genotypes firmly clustered in a monophyletic assemblage with *T. copemani*. A third genotype was almost identical to *Trypanosoma* H25, previously described in an Australian kangaroo, within a clade containing *T. cruzi*, *Trypanosoma marinkellei* and *T. rangeli* – all trypanosomes from South America (Stevens et al., 1999). The relationship supports the hypothesis that this clade may have evolved in ancestral marsupials before the separation of South America, Antarctica and Australia (Hamilton et al., 2009). The five remaining genotypes clustered together in a clade that was most closely related to *T. gilletti*; analysis of a shorter region of the 18S rDNA sequences, including previously described trypanosome sequences from a chuditch and woylies in Western Australia (Smith et al., 2009), placed these genotypes within this clade.

Using a similar nested PCR from the 18S rDNA locus as used in previous studies we found substantially higher levels of trypanosome infection: 67% prevalence in blood from 10 different species of marsupials. In contrast, Paparini et al. (2011) reported a prevalence of only 5% in blood from 11 species of Western Australian marsupials. Within woylies, we found a prevalence of 46% and 88% at Karakamia and the Upper Warren Region respectively, compared to previous detection rates of 14% and 35% for the same areas (Thompson et al., 2008). These differences may be partly attributable to the sensitivity of the PCR used and to the temporal fluctuation of trypanosomes in blood during the natural course of infection. Most of the trapped and released animals screened in the present study may have been in the active acute phase of infection where animals present high parasitemias easily detectable by PCR or microscopy. Previous studies have shown that in the early acute stage of *T. cruzi* infection, diagnosis is straightforward due to high levels of parasitemia but in the chronic stages, low parasitemia often precludes detection in fresh blood (Russomando et al., 1992; Vago et al., 2000; Lane et al., 2003; Campos et al., 2010). Another possible explanation for differences in prevalence may be the demonstrated capacity of Australian trypanosomes to migrate to different organs in the host; in some stages of infection parasites may be more frequently found in tissues than in peripheral blood, reducing the chances of detection by PCR. This could explain why in three woylie carcasses from which we could collect both blood and tissues, trypanosomes were detected by PCR in tissues but not in blood (data not shown). We also found a relatively high prevalence of mixed infections in woylies, but not in other host species (although sample sizes for other hosts were much smaller than for woylies). However, due to the low number of samples examined from the other marsupial species, except blood samples from western grey kangaroos and quolls, we cannot completely exclude the possibility that mixed infections can also occur in other species.

The fact that genotype, clade or species-specific molecular tools were not used previously (Smith et al., 2009; Paparini et al., 2011) may have masked the presence of less abundant trypanosomes in mixed infections. Results of PCR and sequencing using the 18S rDNA generic primers compared with clade-specific primers showed that more abundant Clade A genotypes in mixed infections masked the presence of less abundant Clade B and C genotypes. These results confirm the importance of the use of specific primers for rapid identification of trypanosomes in naturally infected marsupials, especially since sequencing directly from amplicons alone may not be able to detect mixed infections.

Despite PCR results revealed mixed infection in some of the blood samples, only trypanosomes from Clade A were able to be grown in culture. It has been shown that during the isolation and amplification of *T. cruzi* in culture, a selection of genotypes may occur (Bosseno et al., 2000) and that may be the case here. However, cultures from blood samples of animals infected only with trypanosomes from Clade B were unsuccessful indicating that possibly the medias used may not fulfill the nutritional growth needs of Clade B genotypes.

Although all trypanosome genotypes were found in the blood, only some genotypes were seen to colonize tissues. This suggests

a genetic basis for the heterogeneity in the biological behavior of trypanosomes in their marsupial host. We also found, for the first time in trypanosomes infecting Australian marsupials, a differential tissue tropism of trypanosomes within Clades A and B. Several studies have demonstrated that the genetic variability of *T. cruzi* may determine the different histotropism observed during infections and consequently the diverse clinical forms of the disease (Andrade et al., 1999, 2002; Vago et al., 2000; Macedo et al., 2004; da Silva Manoel-Caetano et al., 2008; Ramírez et al., 2010). However, the influence of the host genetic background in this process has been demonstrated as well (Freitas et al., 2009; Andrade et al., 2002). Botero et al. (2007) found a differential tissue tropism of two genetically different clones of *T. cruzi* in experimentally infected mice and Mantilla et al. (2010) reported a mixed infection in a chagasic patient from Colombia, consisting of two genetically diverse *T. cruzi* populations, one of them infecting the oesophagus and the other infecting the heart.

It has been demonstrated that trypanosome multiplication in the vertebrate host occurs extracellularly in the bloodstream or intracellularly in tissues. *T. lewisi* division occurs extracellularly in visceral capillaries, while *T. cruzi* uses host cells to replicate intracellularly and escape from the immune system to continue its life cycle, resulting in the infection of many tissues and organs (De Souza et al., 2010). Our finding of DNA of some genotypes representative of all three trypanosome clades in different marsupial organs, plus the discovery of intracellular amastigotes in some tissues where G2 (Clade A) DNA was present, confirmed that at least this genotype is able to invade cells. It has been shown that intact amastigotes are not easy to find during a chronic *Trypanosoma* infection, possibly due to the damage to host tissues and destruction of intracellular parasites caused by a strong inflammatory process (Higuchi et al., 1993; Zhang and Tarleton, 1999). However, we demonstrated that this genotype was able to invade and replicate in cultured Vero cells, although at a slower rate when compared to *T. cruzi* (data not shown).

Woylies had a greater prevalence of trypanosome infections than any other marsupial species examined and were also the only species found with multiple infections of two or more parasite genotypes. Our study correlates, for the first time, the presence of Australian trypanosomes and pathological changes in woylie tissues, during single infections with parasites belonging to Clade A and also during mixed infections involving two or three trypanosome genotypes. The pattern of inflammatory reactions during these infections was very similar and showed frequent muscle degeneration and occasional necrosis. The pathological changes seen in woylie tissues are similar to those seen in *Didelphis marsupialis* infected with *T. cruzi* from South and North America: myocarditis characterized by inflammatory infiltrates in skeletal muscles and oesophagus (Teixeira et al., 2006). The fact that pathological changes were seen during single and mixed infections involving G2 (Clade A) may merely reflect the virulence of this genotype. However, pathological changes were also observed in mixed infections involving trypanosomes that clustered within Clade B and C (in the absence of Clade A genotypes), suggesting alternative scenarios where the woylie immune system may be less efficient at resolving multiple infections or when mixed infections have potentiated pathogenicity. This latter result is in agreement with a previous study that suggested *T. gilletti* might have the ability to potentiate pathogenicity during concomitant infections in koalas (McInnes et al., 2011b).

Natural mixed infections between different species or genotypes of trypanosomes occur frequently in nature, with both parasite and host factors determining the overall parasitemia, virulence and pathogenicity (Martins et al., 2006; Pinto et al., 2006; Pollitt et al., 2011). Although several studies have reported the intrinsic characteristics of single trypanosome infections, it has not been clearly demonstrated whether the interaction of different trypanosomes in a single host can affect or modify the infection dynamics by either reducing or enhancing parasitemia, virulence and pathogenicity (Reifenberg et al., 1997). Rodrigues et al. (2010) showed that the combination of two strains of *T. cruzi* was able to trigger both protective inflammatory immunity and regulatory immune mechanisms that attenuate damage caused by inflammation in experimentally infected mice. However, in natural infections, virulence is difficult to predict when interactions are not restricted to a single host and parasite, but involve multiple infections as in the case of woylies.

There was a marked difference in the composition of trypanosome infections between woylies in the stable and the declining population. The greater prevalence of genotypes belonging to Clade A and mixed infections in the declining population of woylies, together with the demonstrated potential of these trypanosomes to cause pathological changes in woylies, suggests that infections with trypanosomes within this clade could be important contributors to the dramatic decline of the woylie. Given that we only examined woylies from a single stable population and a single declining population, there is a clear need for further studies to test this hypothesis, although it is noteworthy that *T. copemani* (Clade A) has been reported in the blood of other critically endangered and vulnerable Australian marsupials including Gilbert's potoroos, and quokkas from Western Australia (Austen et al., 2009) and koalas from Queensland (McInnes et al., 2011b).

In summary, this study highlights a wide genetic diversity of trypanosomes within Australian wildlife and reveals for first time the capacity of Australian trypanosomes to infect cells and their pathogenic potential either in single or mixed infections, supporting previous hypotheses (Thompson et al., 2008) associating trypanosome infections with the decline of woylies.

Acknowledgements

Thanks are extended to colleagues at the Department of Environment and Conservation (DEC), Science Division, Manjimup, and to Dr. Manda Page and Alexander Kabat from the Karakamia Sanctuary, for their invaluable help with the collection of blood samples. We thank also Louise Pallant for her helpful advice in the design of the new clade-specific primers and Stephanie Godfrey for her assistance with the presentation of the data. The authors also acknowledge the facilities of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis in the University of Western Australia, a facility funded by the University, State and Commonwealth Governments. This project was supported by funding from the Western Australian Government's State NRM Program.

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Appendix II

Thompson et al. Parasites & Vectors 2013, 6:121
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Parasites & Vectors

RESEARCH Open Access

Morphological polymorphism of *Trypanosoma copemani* and description of the genetically diverse *T. vegrandis* sp. nov. from the critically endangered Australian potoroid, the brush-tailed bettong (*Bettongia penicillata* (Gray, 1837))

Craig K Thompson[1*], Adriana Botero[1], Adrian F Wayne[2], Stephanie S Godfrey[1], Alan J Lymbery[3] and RC Andrew Thompson[1]

Abstract

Background: The trypanosome diversity of the Brush-tailed Bettong (*Bettongia penicillata*), known locally as the woylie, has been further investigated. At a species level, woylies are critically endangered and have declined by 90% since 1999. The predation of individuals made more vulnerable by disease is thought to be the primary cause of this decline, but remains to be proven.

Methods: Woylies were sampled from three locations in southern Western Australia. Blood samples were collected and analysed using fluorescence *in situ* hybridization, conventional staining techniques and microscopy. Molecular techniques were also used to confirm morphological observations.

Results: The trypanosomes in the blood of woylies were grouped into three morphologically distinct trypomastigote forms, encompassing two separate species. The larger of the two species, *Trypanosoma copemani* exhibited polymorphic trypomastigote forms, with morphological phenotypes being distinguishable, primarily by the distance between the kinetoplast and nucleus. The second trypanosome species was only 20% of the length of *T. copemani* and is believed to be one of the smallest recorded trypanosome species from mammals. No morphological polymorphism was identified for this genetically diverse second species. We described the trypomastigote morphology of this new, smaller species from the peripheral blood of the woylie and proposed the name *T. vegrandis* sp. nov. Temporal results indicate that during *T. copemani* Phenotype 1 infections, the blood forms remain numerous and are continuously detectable by molecular methodology. In contrast, the trypomastigote forms of *T. copemani* Phenotype 2 appear to decrease in prevalence in the blood to below molecular detectable levels.

Conclusions: Here we report for the first time on the morphological diversity of trypanosomes infecting the woylie and provide the first visual evidence of a mixed infection of both *T. vegrandis* sp. nov and *T. copemani*. We also provide supporting evidence that over time, the intracellular *T. copemani* Phenotype 2 may become localised in the tissues of woylies as the infection progresses from the active acute to chronic phase. As evidence grows, further research will be necessary to investigate whether the morphologically diverse trypanosomes of woylies have impacted on the health of their hosts during recent population declines.

Keywords: *Trypanosoma vegrandis*, *T. copemani*, Morphology, PCR, Sequencing, Fluorescence, Woylie, *Bettongia*, *penicillata*

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Background

Trypanosomes are parasitic protozoans (Sarcomastigophora: Kinetoplastida), which cause disease and death in humans and livestock around the world. *Trypanosoma cruzi* (Chagas disease) and *T. brucei* (African sleeping sickness) are collectively responsible for about 63,000 human deaths per year [1], while *T. evansi* (Surra), *T. vivax* (Nagana) and *T. congolense* (Nagana) are all of great economic concern to livestock production in Africa, Asia and South America [2-4].

By contrast, the trypanosomes of wildlife have been poorly studied. There is however some evidence indicating that these parasitic protozoans may be the causative agents behind population declines and extinctions of endangered fauna. For example, it was 100 years after a report of the extinction of endemic native rats on Christmas Island before evidence was presented identifying *T. lewisi* as possibly being influential during the disappearance of the native rats [5,6]. Recent studies have shown that the unintentional introduction of the black rat (*Rattus rattus*) and its fleas infected with *T. lewisi* may have contributed to the extinction of *R. macleari* and possibly *R. nativitatis* [5,7].

It is also possible that trypanosomes have played a potential role during the recent decline of the woylie (*Bettongia penicillata*) in Western Australia (WA) [8,9]. Prior to European settlement of Australia in 1788, woylies were distributed over much of the southern half of mainland Australia [10]. However, as a consequence of human expansion and introduced exotic predators, the natural abundance and distribution of the woylie has been severely challenged [10-12]. By the 1970s woylies became restricted to four small populations located in south-western Australia only; namely Tutanning Nature Reserve, Dryandra Woodlands and the Upper Warren Region (UWR) (which includes the Perup and Kingston populations) [11,13] (Figure 1).

The systematic, broadscale control of foxes using 1080-poison began in the 1970's and by 1996 the woylie became the first Australian taxon to have its conservation status downgraded from "Endangered" to "Low Risk / Conservation Dependent" (IUCN Red List) because of recovery efforts [11,12]. However, despite the continuous effort to control exotic predators, woylie populations have undergone a further and rapid decline since 1999 [14-16]. The factors responsible for this recent and rapid decline remain unclear, with the future survival of indigenous woylies becoming increasingly uncertain. However, recent spatio-temporal population modelling has hypothesised that disease, in conjunction with predation, may have been the main contributing factors to the recent woylie population declines [17].

During a recent disease investigation, trypanosomes were identified in the blood of woylies from the UWR [9]. The trypanosomes identified were a morphologically distinct species endemic to Australia, which infected between 35% (by molecular techniques) and 43% (by microscopy) of woylies in these declining populations [9]. In an effort to further investigate the influence of trypanosomes upon the recent decline, research was extended to include woylies at the Karakamia Wildlife Sanctuary (KWS) (Figure 1), as this 285 hectare feral-free enclosure contained the only stable high-density sub-population of woylies on mainland Australia [18]. Molecular techniques identified a trypanosome prevalence of 14% at KWS, with microscopy failing to identify any morphological forms in the blood [9]. The observed higher parasite prevalence and parasitaemia (as interpreted by microscopy) in the declining Upper Warren populations helped strengthen the potential role of disease in the recent woylie decline [9].

The present study extends previous work and examines the morphology of the trypanosomes infecting woylies at both the UWR (with eight woylies translocated to NAR as

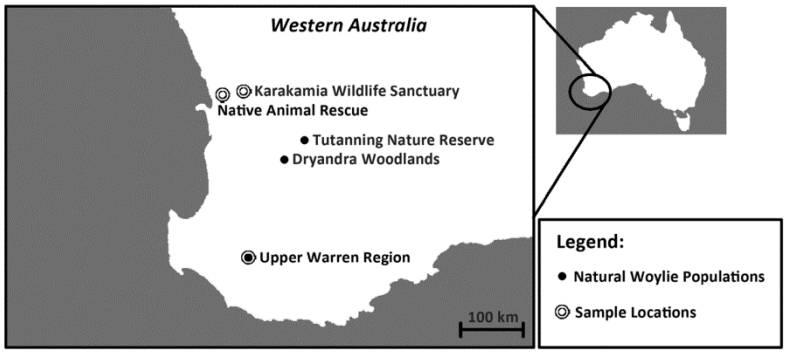

Figure 1 Remaining natural populations of woylies and the sample locations for this study in Western Australia.

part of a more intensive observational study) and at KWS. We report for the first time on two different morphological phenotypes of *T. copemani* infecting woylies. We also report the first visual identification of a second smaller trypanosome, which is believed to be one of the smallest recorded trypanosomes from mammals. The morphology of this new species has been described and named *T. vegrandis* sp. nov. Also presented is the first visual identification of a mixed infection of both *T. copemani* and *T. vegrandis* sp. nov.

Methods

Sample collection and preparation

Sheffield traps baited with a mixture of rolled oats, peanut butter and sardines were used to capture woylies from three separate locations in WA. Firstly from the UWR (Figure 1) during November and December, 2010. UWR is predominantly publicly-owned conservation estate and state forest, managed by the Department of Environment and Conservation (DEC), and supports the largest wild woylie population and two of the four indigenous genetically distinct subpopulations extant at the time [13]. Secondly at Native Animal Rescue (NAR) (Figure 1) on seven separate occasions between April 2011 and April 2012. NAR is managed by the Fauna Rehabilitation Foundation, where a predator proof enclosure (110 m x 70 m) has been purpose-built to house 16 woylies. Thirdly at KWS (Figure 1) on two separate occasions during September 2011 and February 2012. KWS is managed by Australian Wildlife Conservancy, where a predator proof fence has been constructed for native Western Australian endangered mammals.

After removing the animal from the trap, a 400 μl sample of blood was collected from the lateral caudal vein using a 25G x 5/8" needle and 1 ml syringe. From the collected blood, 300 μl was placed into a MiniCollect 1 ml EDTA tube (Greiner bio-one, Germany) to prevent clotting and kept at 4°C for DNA extraction and PCR. With the remaining blood, multiple thin blood smears were made from each woylie sampled. Wet mounted slides were also collected during the February 2012 sampling at KWS. After blood collection, woylies were released at the point of capture, except for eight woylies from the UWR, which were translocated to NAR for release.

Fluorescence *in situ* hybridisation (FISH) and staining

Using *Trypanosoma* Clade B internal forward primer sequence (TVIF [5'- GAC CAA AAA CGT GCA CGT G -3']) [19], a commercially synthesised probe was manufactured which bound an AlexaFluor350 label at the 5' end (BioSynthesis, Texas, USA). The AlexaFluro350 label excites at 350 nm and emits at 442 nm.

The FISH protocol used in this study was modified from that developed by Li [20] and was conducted within 24 hours of blood collection. After application of a 125 μl Frame-Seal Incubation Chamber (Bio-Rad, California, USA) to the dried blood slide, cells within the chamber were fixed with 120 μl of 4°C fixative buffer (88 μl of 95% ethanol, 20 μl of deionised H_20 and 12 μl of 25x SET buffer [3.75 M NaCl, 25 mM EDTA, 0.5 M Tris HCl @ pH 7.8]). The buffer was left to incubate at 4°C for 40 minutes. The fixative buffer was then drained from the chamber using filter paper and washed with PBS (950 ml of distilled H_2O, and 50 ml of 20x PBS stock solution [160.0 g/L of NaCl, 24.2 g/L of KH_2PO4 and 6.8 g/L of K_2HPO_4]). After drying the slide in an incubation oven at 58°C for 15 minutes, the cells within the chamber were dehydrated using 50%, 80% and 96% ethanol steps for a period of 90 seconds each, after which, the slide was allowed to air dry.

The remaining steps of the FISH protocol were conducted in a darkened room. To the slide chamber, 125 μl of hybridisation mix (2 μl of TVIF probe (20 μM), 5x SET buffer, Igepal-CA630 [Sigma, Castle Hill, NSW, Australia] and 25 μg/ml polyA potassium salt [Sigma, Castle Hill, NSW, Australia]) was added and the slide placed into an incubation oven at 58°C for 90 minutes. The hybridisation mix was removed from the chamber with filter paper and the cells within the chamber were washed with 1x SET buffer preheated to 58°C. An additional 125 μl of the preheated 1x SET buffer was added to the slide chamber and the slide placed into an incubation oven at 58°C for 15 minutes; this step was repeated once more. The 1x SET buffer was drained out of the chamber with filter paper and the frame-seal removed. The slide was allowed to air dry in the darkened room.

Once dried, the slide was then stained with Modified Wright's stain in the darkened room and allowed to dry again. Due to an auto-fluorescence issue with anti-fading agents and the Modified Wright's stain, a drop of distilled water was placed onto the slide and covered with a 50 mm cover slip. The edges of the cover slip were sealed with clear nail-polish to prevent evaporation. The slide was then placed into a dark storage box until viewed under the microscope.

Microscopy and image acquisition

The hybridised and stained slides were examined with a BX51 microscope (Olympus, Japan) using white light, as well as ultraviolet light (330 – 385 nm) through an emission filter (420 nm). The Alexafluor350 probe fluoresced bright blue under the ultraviolet light conditions. Slides were scanned using the 40x objective lens, with digital images captured using the 100x objective lens. Digital images with an inserted scale bar were captured in a TIFF file format using a microscope mounted camera and DP Controller (Olympus, Japan). Measurements of the key morphological features as described by Hoare

part of a more intensive observational study) and at KWS. We report for the first time on two different morphological phenotypes of *T. copemani* infecting woylies. We also report the first visual identification of a second smaller trypanosome, which is believed to be one of the smallest recorded trypanosomes from mammals. The morphology of this new species has been described and named *T. vegrandis* sp. nov. Also presented is the first visual identification of a mixed infection of both *T. copemani* and *T. vegrandis* sp. nov.

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[21] were made using Adobe Photoshop CS5 Extended (Adobe Systems Incorporated, USA).

Morphology

Key morphological measurements were recorded for each of the trypanosomes observed. Morphological traits recorded included total length (L) (including free flagellum), width over the nucleus (W), distance of the posterior to kinetoplast (PK), posterior to nucleus (PN), kinetoplast to nucleus (KN), anterior to nucleus (NA) and length of the free flagellum (FF). For *T. copemani* the kinetoplast-length (K-l) and kinetoplast-width (K-w) were also measured.

Two additional ratios were calculated as they have been used previously to discriminate species of trypanosomes [21]; the Nucleus Index (NI) (=PN/NA) and the Kinetoplast Index (KI) (=PN/KN). When NI=1, the nucleus is in the middle of the body; NI<1, the nucleus is posteriorly located in the body; and N1>1, the nucleus is anteriorly located in the body [21]. When KI=2, the kinetoplast is half way between the posterior and nucleus; KI<2, the kinetoplast is closer to the posterior than to the nucleus; and KI>2, the kinetoplast is closer to the nucleus than the posterior end of the body [21].

Differences in morphology between groups of trypanosomes were tested over all morphological traits (except the two ratio traits) with multivariate analysis of variance (MANOVA), and differences between groups for each trait (including the ratio traits) were tested by single factor analyses of variance, using a Bonferroni correction to maintain an experiment-wide Type I error rate of 5%. If the MANOVA showed a significant difference between groups, then discriminant analysis was used to detect the best combination of traits separating the groups. All statistical analyses were conducted with the software JMP 10.0 [22].

DNA Extraction

Blood collected in EDTA tubes were used for genomic DNA extraction. DNA was extracted from 300 μl of host blood using the Wizard® Genomic DNA Purification Kit (Cat# A1125) as per the protocol for whole blood extraction (Promega, Wisconsin USA). DNA was eluted in 60 μl of DNA Rehydration Solution and stored at −20°C prior to use. A negative control was included in each batch of DNA extractions, which contained no blood.

Clade-specific PCR

Three separate clade-specific nested PCR protocols were used to amplify the trypanosome 18S rDNA region using primers and PCR reactions as previously described [19,23]. However, this study used different PCR conditions for four of the primer pairs. For *T. copemani* external primers (S825F and SLIR) [19], the pre-PCR step was 1 cycle of 94°C for 5 mins, 50°C for 2 mins and 72°C for 4 mins, followed by 35 cycles of 94°C for 30 secs, an annealing temperature of 57°C for 30 secs and an extension temperature of 72°C for 2 mins 20 secs, with a final step of 72°C for 7 mins. For *T. copemani* internal primers (WoF and WoR) [23] and Clade B external and internal primers (TVEF, TVER, TVIF, TVIR) [19], the annealing temperature for the 35 cycles was 58°C for 30 secs, while the extension temperature was the same but held for only 50 seconds per cycle. *T. gilletti* species-specific primer sets were used as previously described [23].

Four controls were used in every nested PCR and included the negative control from the DNA extraction, a primary and a secondary PCR negative control and PCR positive control. All were monitored to ensure reliability of results. PCR products were run on a 1.5% agarose gel using SYBR Safe Gel Stain (Invitrogen, California USA) and visualized by illumination with UV light.

Sequencing PCR

A fourth nested PCR was used to amplify positive samples of *T. copemani* and *T. vegrandis* sp. nov. for sequencing. This technique targeted the 18S rDNA region and used the primers and PCR reaction as previously described [19] but with different PCR conditions for each of the three primer pairs. For the external primers SLF and S762R, the pre-PCR step was 1 cycle of 94°C for 5 mins, 50°C for 2 mins and 72°C for 4 mins, followed by 35 cycles of 94°C for 30 secs, an annealing temperature of 55°C for 30 secs and 72°C for 2 mins 20 secs, with a final step of 72°C for 7 mins. For the first pair of internal primers S823F and S662R, the annealing temperature for the 35 cycles was 56°C for 30 secs, while for the second pair of internal primers S825F and SLIR, the annealing temperature for the 35 cycles was 57°C for 30 secs. The combination of these two PCR products amplified a 1410 bp amplicon for sequencing. Four controls were used as described above. PCR products were run on a 1.5% agarose gel using SYBR Safe Gel Stain (Invitrogen, California USA) and visualized by illumination with UV light.

PCR products of appropriate size were purified using the Agencourt AMPure PCR Purification system (Beckman Coulter, California USA) as per the manufacturer's instructions and sequenced using an ABI Prism™ Terminator Cycle Sequencing Kit (Applied Bio-systems, California USA) on an Applied Bio-System 3730 DNA Analyser. The resulting sequences were then analysed and aligned using ClustalX 2.1.

Results

Trypanosoma copemani- microscopy and image acquisition

During the trapping sessions at the UWR, eight of the 15 blood samples examined by microscopy were identified positive for *T. copemani* infections. From these eight

infected woylies, a total of 110 trypanosomes were identified in the blood smears. All were trypomastigote forms and their overall measurements are presented in Table 1. The trypomastigotes observed were both broad (N=78) (Figure 2A) and slender (N=32) (Figure 2B). No divisional stages of the trypanosomes were observed in the blood smears. These same eight positive woylies were translocated to the purpose built enclosure at NAR and were part of the temporal study undertaken there.

When considering the phylogenetic analysis presented by Botero *et al.*, [19] and the sequencing results below, the 110 trypomastigotes measured were separated into two groups. The first group was "*T. copemani* Phenotype 1" (P1) comprising trypomastigotes found in Woylie ID: WC2741, WC2830, WC2842, WC2844 & WC2920 and the second was "*T. copemani* Phenotype 2" (P2) comprising trypomastigotes found in Woylie ID: WC2807, WC2841 & WC2930. Mean morphological traits for *T. copemani* P1 (Figure 3A) and *T. copemani* P2 (Figure 3B) are shown in Table 1.

From the MANOVA there was a significant morphological difference between groups ($F_{9,100}$ = 22.06, P < 0.0001). Univariate tests found significant differences between groups in KN ($F_{1,108}$ = 131.42), L ($F_{1,108}$ = 34.10), KI ($F_{1,108}$ = 22.73), K-I ($F_{1,108}$ = 13.98), PN ($F_{1,108}$ = 12.11) and NA ($F_{1,108}$ = 10.60) (P < 0.05 for all tests, with the Bonferroni). Discriminant analysis correctly classified 95.5% of the original grouped cases along one canonical discriminant function, which loaded most heavily for KN (0.78), L (0.40), K-I (−0.26), PN (0.24) and NA (0.22).

Trypanosoma copemani- PCR and sequencing

The clade-specific PCR confirmed the presence of *T. copemani* in the eight woylies at the time of translocation from the UWR to NAR. Two distinct genotypes were also identified by sequencing, having a 13 base-pair variation over the larger combined 1410 bp amplicon. The grouping of the eight infected woylies based on the two different genotypes was the same as the phenotypic grouping presented above.

The temporal molecular analysis of infected woylies at NAR between April 2010 and April 2011 indicated that the five woylies infected with *T. copemani* P1 remained PCR positive throughout the study period (Table 2). The November 2011 and April 2012 positive samples were sequenced and confirmed the continued presence of *T. copemani* P1. On the one and only occasion PCR failed to identify the presence of *T. copemani* in these five woylies (WC2842 during Dec 2011; < 50 μl of blood was collected from this woylie on this occasion) microscopy was used alone to confirm the morphological presence of two trypomastigotes on the blood slides.

In contrast, of the three woylies infected with *T. copemani* P2, two of them (WC2807 and WC2930) lost the blood form of the parasite to below PCR detectable levels in the full 300 μl of blood from March and April 2012 (Table 2). These negative PCR results were supported by the absence of trypomastigotes in the thin blood smears when examined by microscopy. In an effort to strengthen the reliability of these negative results from March and April 2012, nine additional nested PCR reactions were performed on each of the DNA extractions from WC2807 and WC2930. All 40 nested PCR reactions returned a negative result. The November 2011 and April 2012 positive samples were sequenced and confirmed the continued presence of *T. copemani* P2.

Trypanosoma vegrandis sp. nov. - microscopy and image acquisition

During sampling at KWS in February 2012, wet blood mounts were made from four woylies previously sampled in September 2011 and shown by PCR to be mono-infected with *T. vegrandis* sp. nov. The movement

Table 1 Morphological traits of the trypomastigotes of *T.copemani*: mean measurements (μm) ± standard error (range)

Measurements (μm)	Overall (N=110)	P1 (N=82)	P2 (N=28)
L	37.34 ± 0.33 (30.25 - 45.19)	36.35 ± 0.35 (30.25 - 45.19)	40.26 ± 0.53 (33.41 - 43.84)
W	6.12 ± 0.18 (1.15 - 10.23)	5.98 ± 0.21 (1.51 - 10.23)	6.53 ± 0.31 (3.71- 9.24)
PK	11.44 ± 0.22 (3.93 - 15.89)	11.49 ± 0.27 (3.93 - 15.89)	11.27 ± 0.35 (5.43 - 14.79)
PN	15.42 ± 0.23 (7.34 - 19.53)	14.98 ± 0.27 (7.34 -19.04)	16.71 ± 0.32 (11.10 - 19.53)
KN	4.36 ± 0.10 (2.52 - 7.31)	3.92 ± 0.07 (2.52 - 5.80)	5.66 ± 0.17 (4.19 - 7.31)
NA	15.85 ± 0.23 (9.30 - 22.06)	15.44 ± 0.26 (9.30 - 22.06)	17.06 ± 0.36 (13.15 - 20.91)
NI	0.98 ± 0.02 (0.39 - 1.42)	0.98 ± 0.02 (0.39 - 1.42)	0.99 ± 0.03 (0.60 - 1.37)
KI	3.64 ± 0.08 (1.38 - 5.96)	3.85 ± 0.09 (1.38 - 5.96)	3.02 ± 0.10 (1.96 - 4.20)
FF	8.24 ± 0.19 (3.44 - 12.39)	8.17 ± 0.22 (3.44 - 12.39)	8.44 ± 0.32 (4.38 - 12.35)
K-I	1.02 ± 0.02 (0.61 - 1.31)	1.06 ± 0.02 (0.76 - 1.31)	0.92 ± 0.04 (0.61 - 1.25)
K-w	0.71 ± 0.01 (0.50 - 1.00)	0.72 ± 0.01 (0.50 - 1.00)	0.69 ± 0.02 (0.50 - 0.93)

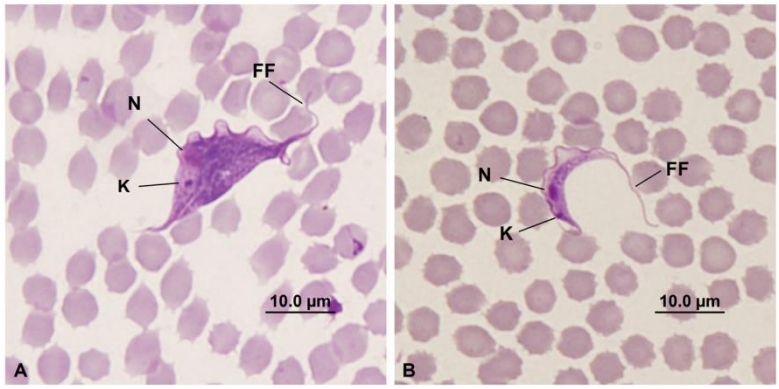

Figure 2 The different morphological forms of *T. copemani* identified from the same host. A - Broad trypomastigote form. **B** - Slender trypomastigote form. K= Kinetoplast, N= Nucleus, FF= Free Flagellum.

of this novel motile flagellated trypanosome was observed by microscopy as being a characteristic "corkscrew type" action.

A total of 20 trypanosomes were identified in stained blood smears from these same four woylies (Woylie ID: 7199222, 7236356, 7225370 & K734) and measured. All were identified as trypomastigote forms (Figure 4). The overall measurements of these 20 trypomastigotes are presented in Table 3.

When considering the phylogenetic analysis presented by Botero *et al.*, [19] and the sequencing PCR results below, the 20 trypomastigotes measured were separated into two groups. The first group was "*T. vegrandis* sp. nov. Genotype 1" (G1), comprising trypomastigotes found from Woylie ID: 7199222 & 7236356 and the second was "*T. vegrandis* sp. nov. Genotype 2" (G2) comprising trypomastigotes found in Woylie ID: 7225370 & K734. Mean morphological traits for each group are shown in Table 3. There were no significant differences between groups using all morphological traits in MANOVA ($F_{7,12}$ = 0.97, P = 0.49) or using each trait separately in univariate ANOVA's. This genetically varied clade appears to be represented by a single, morphologically unique phenotype (Figure 4).

During the April 2012 sampling at NAR, blood mounts from one woylie known to be PCR positive for infections with *T. vegrandis* sp. nov. (WC2741; Table 4) were successfully hybridised and stained. Three trypomastigotes were identified by the hybridisation of the fluorescence probe *in situ* (Figure 5). The morphological measurements of these three trypomastigotes are presented in Table 3.

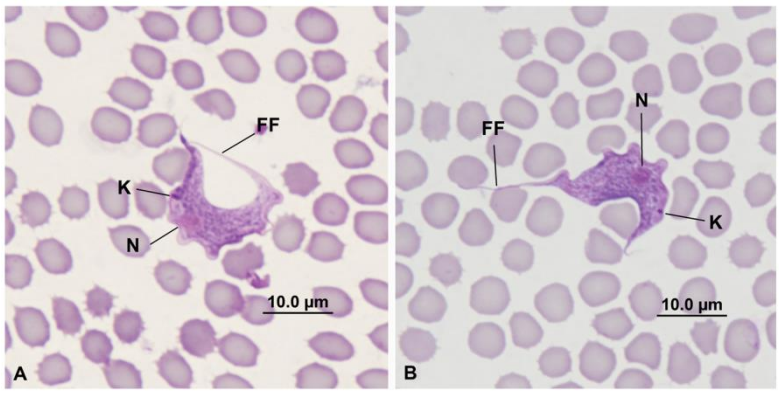

Figure 3 The different morphological phenotypes of *T. copemani* identified from different hosts. A - *T. copemani* Phenotype 1 (P1). **B** - *T. copemani* Phenotype 2 (P2). K= Kinetoplast, N= Nucleus, FF= Free Flagellum.

Table 2 PCR results showing the presence of *T. copemani* P1 and P2 tested at NAR between April 2011 and April 2012

Woylie ID number	Trypanosome species	April 2011	June 2011	July 2011	Nov 2011	Dec 2011	March 2012	April 2012
WC2741	*T. copemani* P1	+	+	+	+	+	+	+
WC2830	*T. copemani* P1	+	+	+	+	+	+	+
WC2842	*T. copemani* P1	+	+	+	+	-*		+
WC2844	*T. copemani* P1		+	+	+	+	+	+
WC2920	*T. copemani* P1	+	+	+	+	+	+	+
WC2807	*T. copemani* P2	+	+	-	+	+	-	-
WC2841	*T. copemani* P2	+	+	+	+	+	+	+
WC2930	*T. copemani* P2	+	+	+	+	+	-	-

[+] PCR positive for *T. copemani*.
[–] PCR negative for *T. copemani*.
[ ] Woylie not trapped that month.
[*] PCR negative but positive to *T. copemani* by microscopy.

Trypanosoma vegrandis sp. nov. - PCR and sequencing

The clade-specific nested PCR confirmed the presence of *T. vegrandis* sp. nov. in four woylies at KWS (Woylie ID: 7199222, 7236356, 7225370 & K734) during the trapping sessions of September 2011 and February 2012. These same four woylies tested negative to *T. copemani* and *T. gilletti*. Two distinct genotypes were identified by the sequencing of the PCR products, the first group was "*T. vegrandis* sp. nov. G1", again comprising trypomastigotes found in Woylie ID: 7199222 & 7236356 (representing G3 and G6 genetic sequences of Clade B [19]). The second group was "*T. vegrandis* sp. nov. G2", again comprising trypomastigotes found in Woylie ID: 7225370 & K734 (representing G4, G5 and G7 genetic sequences of Clade B [19]).

Of the five *T. copemani* P1 positive woylies at NAR, three of them (WC2741, WC2844 and WC2920) were also PCR positive for *T. vegrandis* sp. nov. Both WC2741 and WC2844 maintained a consistent mixed infection during the 13 month sampling period (Table 4). Of the three woylies infected with *T. copemani* P2, there was only one occasion that *T. vegrandis* sp. nov. was detected by the species-specific PCR as a mixed infection within the blood; this being WC2841 in December 2011 (Table 4).

All attempts to amplify *T. vegrandis* sp. nov. in the mixed presence of *T. copemani* using the sequencing PCR protocol failed. Also all attempts to amplify *T. vegrandis* sp. nov. with the *T. gilletti* species-specific PCR protocol failed.

Description of a new species- *Trypanosoma vegrandis* sp. nov.

Based on results presented here we propose the name *Trypanosoma vegrandis* sp. nov. for this morphologically and genetically distinct species found within the woylie.

Diagnosis: Morphological analysis of various blood forms from the woylie or Brush-tailed Bettong (*B. penicillata*) including microscopy of live motile bodies, fluorescence *in situ* hybridisation and Modified Wright's staining of fixed trypomastigotes. Description represents a single phenotype

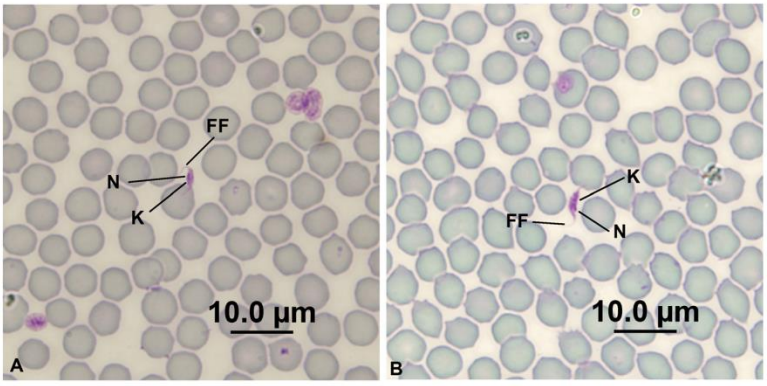

Figure 4 The morphological form of *T. vegrandis* sp. nov. A and **B** - Trypomastigote forms. K= Kinetoplast, N= Nucleus, FF= Free Flagellum.

Table 3 Morphological traits of the trypomastigotes of *T. vegrandis* sp. nov.: mean measurements (μm) ± standard error (range)

Measurements (μm)	Overall (N=20)	G1 (N=10)	G2 (N=10)	Genotype unknown FISH (N=3)
L	8.30 ± 0.28 (6.92 - 10.50)	8.12 ± 0.31 (6.92 - 10.25)	8.47 ± 0.47 (7.02 - 10.50)	8.85*
W	1.33 ± 0.04 (1.00 – 1.63)	1.33 ± 0.06 (1.04 – 1.63)	1.33 ± 0.05 (1.00 – 1.57)	1.37± 0.03 (1.32 – 1.41)
PK	3.26 ± 0.09 (2.71 - 3.87)	3.30 ± 0.12 (2.78 - 3.87)	3.22 ± 0.14 (2.71 - 3.87)	2.95± 0.12 (2.81 – 3.18)
PN	4.39 ± 0.15 (3.28 – 5.68)	4.28 ± 0.15 (3.28 – 4.78)	4.49 ± 0.26 (3.46 – 5.68)	4.19± 0.16 (3.96 – 4.49)
KN	1.22 ± 0.07 (0.85 – 1.95)	1.11 ± 0.05 (0.95 – 1.39)	1.34 ± 0.12 (0.85 – 1.95)	1.24± 0.05 (1.15 – 1.31)
NA	2.16 ± 0.11 (1.56 – 3.27)	2.02 ± 0.12 (1.56 – 2.87)	2.30 ± 0.17 (1.76 – 3.27)	2.62± 0.10 (2.47 – 2.82)
NI	2.10 ± 0.09 (1.42 – 2.80)	2.18 ± 0.13 (1.59 – 2.80)	2.01 ± 0.13 (1.42 – 2.61)	1.61± 0.11 (1.46 – 1.82)
KI	3.70 ± 0.15 (2.70 – 4.95)	3.92 ± 0.21 (3.24 – 4.94)	3.47 ± 0.19 (2.70 – 4.95)	3.37± 0.06 (3.25 – 3.44)
FF	1.86 ± 0.10 (1.24 – 2.88)	1.83 ± 0.17 (1.28 – 2.88)	1.89 ± 0.13 (1.24 – 2.57)	2.40*

* Sample Number = 1.

encompassing two different genotypes identified by phylogenetic analysis of the 18S rDNA and gGAPDH gene [19].

The trypomastigotes of *T. vegrandis* sp. nov. have a curved body which is drawn out to a pointed posterior end. They are a small trypanosome with a smallest recorded length being 6.92 μm. The width of the trypanosome is about 16% that of its total length and the free flagellum is relatively long, being over 20% of the total length. The nucleus is located in the anterior half of the body, with the posterior kinetoplast positioned closer to the nucleus than to the posterior edge of the body. The distance between the nucleus and kinetoplast is about 20% of the body length, when excluding the free flagellum.

The mean total length of the trypomastigotes found in the blood of the woylie was 8.30 μm (range: 6.92 - 10.50 μm), mean width- 1.33 μm (range: 1.00 - 1.63 μm), mean posterior edge to kinetoplast distance- 3.26 μm (range: 2.71 - 3.87 μm), mean posterior edge to nucleus distance- 4.39 μm (range: 3.28 - 5.68 μm), mean kinetoplast to nucleus distance- 1.22 μm (range: 0.85 - 1.95 μm), mean nucleus to anterior edge distance- 2.16 μm (range: 1.56 - 3.27 μm) and mean free flagellum length- 1.86 μm (range: 1.24 - 2.88 μm). The mean NI index was 2.10 μm (range: 1.42 - 2.80 μm) and mean KI index was 3.70 μm (range: 2.07 - 4.95 μm).

Taxonomic summary

Vertebrate type host Brush-tailed Bettong (*Bettongia penicillata*)

Vertebrate additional hosts Western Grey Kangaroo, Quenda, Tammar Wallaby, Chuditch [19]

Invertebrate vector Unknown

Morphological type location Karakamia Wildlife Sanctuary (S31.82073; E116.24604)

Table 4 PCR results showing the presence of *T. vegrandis* sp. nov. tested at NAR between April 2011 and April 2012

Woylie ID number	Trypanosome species	April 2011	June 2011	July 2011	Nov 2011	Dec 2011	March 2012	April 2012
WC2741	*T. vegrandis* sp. nov.	+	+	+	+	+	+	+
WC2830	*T. vegrandis* sp. nov.	-	-	-	-	-	-	-
WC2842	*T. vegrandis* sp. nov.	-	-	-	-	-		-
WC2844	*T. vegrandis* sp. nov.		+	+	+	+	+	+
WC2920	*T. vegrandis* sp. nov.	+	+	+	-	-	+	+
WC2807	*T. vegrandis* sp. nov.	-	-	-	-	-	-	-
WC2841	*T. vegrandis* sp. nov.	-	-	-	-	+	-	-
WC2930	*T. vegrandis* sp. nov.	-	-	-	-	-	-	-

[+] PCR positive for *T. vegrandis* sp. nov.
[–] PCR negative for *T. vegrandis* sp. nov.
[ ] Woylie not trapped that month.

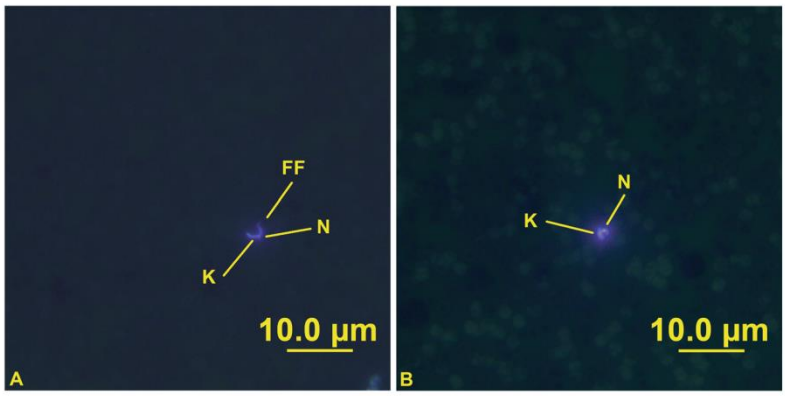

Figure 5 Fluorescence in situ hybridisation of *T. vegrandis* sp. nov. A and **B** - Fluorescent trypomastigote forms. K= Kinetoplast, N= Nucleus, FF= Free Flagellum.

Additional locations Upper Warren Region (S34.11528; E116.32362), Native Animal Rescue (S31.86677; E115. 89072) and Dwellingup, WA

Site of infection Blood, brain (results not shown) as well as skeletal muscle, heart, lung, oesophagus, tongue, kidney, bone marrow, liver and spleen [19]

Pre-patent and patent periods Unknown

Subacute phase Unknown

Chronic phase Unknown

Etymology This species has been given the name *vegrandis* due to the small size; vegrandis is a logical name as it means diminutive, small and tiny.

Mixed Infection- microscopy and image acquisition

The mixed infection of *T. copemani P1* and *T. vegrandis* identified by molecular methodology for Woylie ID: WC2741 at NAR was confirmed by microscopy in April 2012 during the hybridization and staining procedure. When the fluorescent conditions of Figure 5B were changed to white light microscopy, the field of view contained not only the hybridised *T. vegrandis* (Figure 6- circle) but also contained the thin trypomastigote of *T. copemani* P1 (Figure 6- arrow). Figures 4 and 6 also illustrate the varying translucency of the *T. vegrandis* trypomastigote forms when stained. The absence of fluorescence in the vicinity of *T. copemani* in Figure 5B confirmed the specificity of the probe.

Discussion

Overall, *T. copemani* trypomastigotes in the blood of woylies were characterised by a long curved body. The nucleus, on average, was near the centre of the body and the posterior, oval-shaped kinetoplast was positioned closer to the nucleus than to the posterior edge of the body. A free flagellum was present and was about 20% of the total length. The two phenotypes of *T. copemani* were distinguished by the statistically significant differences in a number of morphological traits, in particular the distance between the nucleus and kinetoplast. The KN distance for P1 was 3.92 ± 0.07 µm and for P2 was 5.66 ± 0.17 µm. The different

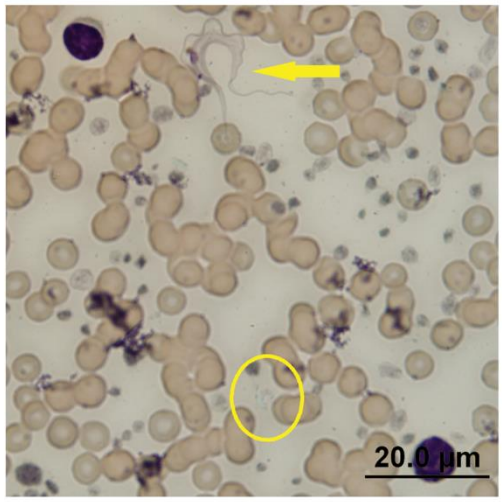

Figure 6 Trypomastigotes of *T. copemani* P1 (arrow) and *T. vegrandis* (circle) from NAR.

KN distances were not believed to be an artefact as all blood smears were made in exactly the same manner and the phenotypic grouping of the eight woylies at NAR matched exactly with the genotypic grouping of these same woylies.

From the *T. copemani* measurements, we suggest that the different thicknesses observed represent different life stages of the trypanosome within the host, as both broad and slender forms were observed simultaneously within individual P1 and P2 infected hosts. We believe that the broad trypomastigote was the blood form responsible for the reproductive phase (71% of trypomastigotes measured) and the slender form was the adult trypomastigote. Similar size variations of the trypomastigote blood forms have been observed in various mammalian hosts and includes *T. lewisi, T. musculi, T. evotomys* and *T. zapi* [21]. Varying trypomastigote thickness was also observed in the Gilbert's Potoroo (*Potorous gilbertii*), with *T. copemani* trypomastigotes grouped as slender, medium and broad [24].

There appears to be host-induced morphological variation of trypanosomes found infecting wildlife. The morphology of *T. copemani* observed in the woylie, for example, differs to that of *T. copemani* found in Gilbert's potoroo, being relatively longer and thinner. The smallest length recorded in the woylie was 30.25 μm, while in the potoroo it was 25.0 μm and the widest length recorded in the woylie was 10.23 μm, while in the potoroo it was 15.4 μm [24]. Other dissimilarities included a larger PK mean in the woylie (11.44 μm compared to 8.1 μm in the potoroo), a smaller KN and FF mean in the woylie (4.36 μm and 8.24 μm compared to 5.8 μm and 10.8 μm respectively in the potoroo) [24]. Also dividing trypomastigotes of *T. copemani* were identified in the potoroo [24], whereas we failed to locate any divisional forms in the woylies. Compared to *T. copemani* in the quokka (*Setonix brachyurus*), trypomastigotes of *T. copemani* in the woylie were wider (6.16 μm compared to 4.2 μm in the quokka), had a larger PK and NA mean (11.44 μm and 15.85 μm compared to 6.5 μm and 13.7 μm respectively in the quokka) and a smaller KN and FF mean (4.36 μm and 8.24 μm compared to 5.9 μm and 12.1 μm respectively in the quokka) [24]. This polymorphism emphasises the importance of using both morphological and genetic criteria in describing trypanosomes from wildlife.

The two morphological phenotypes of *T. copemani* described in the present study, P1 and P2, correspond to Clade A Genotypes 1 and 2 respectively, of which only P2 (≈ G2) has the ability to invade and divide within the cells of the host [19]. Two woylies, which were sampled over time at NAR (WC2807 and WC2930), appeared to lose their infection with *T. copemani* P2; this was based on the lack of PCR detection of trypanosomes in the peripheral blood. This may be because the infection became localised to the tissues of the host. In contrast, woylies infected with *T. copemani* P1, including those concurrently infected with *T. vegrandis* consistently maintained detectable levels of *T. copemani* P1 during this study. Due to our small samples sizes for these observations, further work is required to determine if *T. copemani* P1 is capable of infecting tissues, which may indicate a significant difference in virulence potential between the two forms of *T. copemani*.

The chronic effect of *T. copemani* P2 within the woylie remains unknown. However, it has been hypothesised from histopathological observations that when *T. copemani* P2 invade host cells as part of the life cycle in the woylie, it may initiate a strong inflammatory response of the host, with significant tissue degeneration occurring in the heart, oesophagus, kidney and tongue [19]. The pathological lesions and tissue degeneration observed within infected woylies with *T. copemani* P2 show similarities to the pathological changes observed in infected opossums with *T. cruzi* [19,25]. Overtime these pathological changes to the woylies may reduce its fitness and be a contributing factor during its recent decline.

The naming of *T. vegrandis* was supported by microscopic visualisation of live motile stages, Modified Wright's stained trypomastigotes and hybridised forms stained with a species-specific fluorescent probe. It is also complemented by the genetic amplification using species-specific Clade B primers, the failure of the *T. vegrandis* species-specific fluorescent probe to hybridise to *T. copemani* (Figure 5B & 6), and failure of genetic amplification using the *T. gilletti* species-specific primers.

In spite of the genetic variability seen in the phylogenetic analysis of Clade B by Botero *et al.*, [19] we observed morphological uniformity, grouping the genetic sequences of Clade B G3 – G7 together as a single morphological phenotype. This provides further support that the description of a trypanosome species should not be based on morphology alone, due to the polymorphic nature of the trypomastigotes in the blood (as discussed above with *T. copemani* in different hosts). As such, further studies are required to determine whether *T. gilletti* has morphological affinities with *T. vegrandis*, since *T. gilletti* was described solely on genetic data [26].

The morphology of *T. vegrandis* has been elusive since its molecular identification and in hindsight, is not surprising that it was not detected in previous studies when its small size and translucent nature are taken into account. We now know that *T. vegrandis* is the most prevalent trypanosome within this sub-population of woylies at KWS (unpublished data) but may well have been overlooked in previous surveys at KWS [9] and other locations [8,27], because of the limitations of the molecular tools used at the time.

Another reason for the absence of previous morphological observations may be the critical timing between blood collection and fixation of the slide. We believe that the slides need to be fixed and stained within the 24 hour period after blood collection. Increasing the time for fixation of the blood slide results in a degradation of *T. vegrandis*, to a point where it is no longer detectable by microscopy (unpublished data). This was the case for samples collected at KWS in September 2011 where 22 PCR-positive blood smears were collected, and were fixed and stained four weeks later. By this time no morphological forms of *T. vegrandis* were identified by microscopy. It is therefore possible that this may have been the case for the molecular-based reports of *T. gilletti* from koalas and *T. gilletti*-like trypanosomes from woylies where no morphological forms were identified [26,27].

Overall, the trypomastigotes of *T. vegrandis* in the blood of woylies were approximately 20% the length of *T. copemani*, with a minimum length of 6.92 μm. The previous smallest reported individual trypomastigote lengths that we found were for *T. congolense* and *T. simiae*, both with a minimum length of 8 μm [21]. *T. vegrandis* is believed to be one of the smallest trypanosomes reported infecting mammals [21,24,26,28-37]. Using the smallest length of both *T. vegrandis* and *T. copemani* from this study and the comprehensive analysis of trypanosome species complied by Hoare [21], Figure 7 compares the smallest individual recorded length for each sub-genus, as well as the mean of the smallest recorded lengths of the species within each sub-genus (N=104).

T. copemani was grouped within the subgenus *Herpetosoma* due to the long free flagellum, oval kinetoplast, and the relatively large distance between the kinetoplast and nucleus [24]. Surprisingly, similar morphological ratios of *T. vegrandis* are reported here. Apart from the very small size of *T. vegrandis*, all of the body proportions (except for the NI index) are similar to that of *T. copemani*, with a relatively long free flagellum and a relatively large distance between the kinetoplast and nucleus. At this stage it is very difficult to assign *T. vegrandis* to a subgenus. The phylogenetic analysis of the 18S rDNA and *gGAPDH* genes suggest that *T. vegrandis* may be part of the Stercoraria grouping of trypanosomes as it shares a close evolutionary relationship with *T. copemani* and *T. pestanai* (both being part of the Stercoraria group) [19,21]. Further work is required to understand the transmission dynamics from vector to host, along with the life cycle of the trypanosome in the vector before this can be commented on further.

Conclusion

In this report we describe the morphological polymorphism of *T. copemani*, which includes the different trypomastigote phenotypes from the blood of woylies. We also provide the first morphological observations and taxonomic description of trypanosomes from a new genetically diverse clade, for which we propose the name *T. vegrandis*. Up until now this small trypanosome has only been identified by PCR from a variety of hosts, including the woylie. Using fluorescence *in situ* hybridisation and light microscopy, we described a mixed trypanosome infection in a woylie, with both *T. copemani* and *T. vegrandis* observed. The temporal reduction of *T. copemani* P2 in the peripheral blood of the woylie and its ability to invade cells may

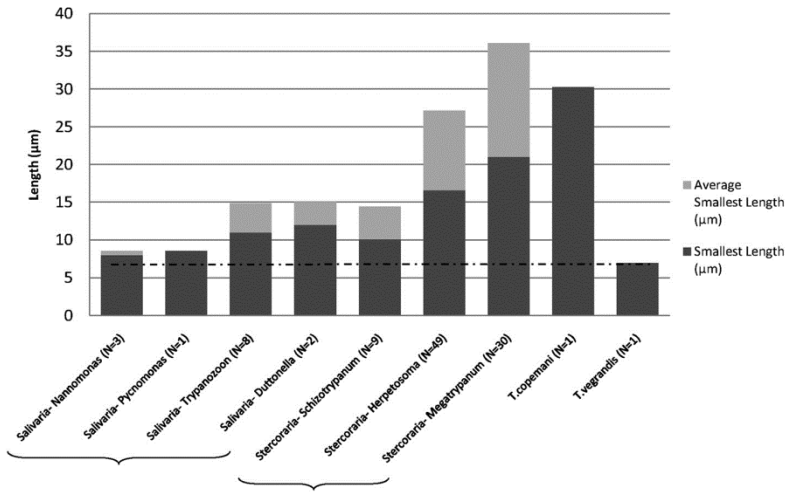

Figure 7 Smallest reported length and average smallest length of the species within each subgenus of *Trypanosoma* [21], *T. copemani* and *T. vergrandis*.

suggest that this more virulent phenotype could become localised within the tissues of the host. Over time, tissue degeneration of the host could result in an overall reduced fitness, making the woylie more susceptible to predation in the wild.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
CKT, AB, AFW and RCAT designed the study; CKT, AFW, SSG, AJL and RCAT implemented the study; CKT managed the data; CKT, AB, SSG and AJL analysed and interpreted the data; CKT wrote the paper. CKT, AFW, SSG and RCAT supervised the different phases of the study. All authors read, revised and approved the final manuscript.

Acknowledgements
We would like to thank Manda Page, Jo Kuiper and Alexander Kabat at KWS, Marika Maxell, Chris Vellios and Colin Ward at DEC Science Division, Manjimup, Lizzie Aravidis and Kelli Ellemor at NAR, as well as all of the volunteers for their assistance in the collection of samples. We would also like to thank Andrew Li and Peter Adams for their assistance with developing the FISH protocol and Louise Pallant for her guidance with molecular techniques. Funding for this project was supplied to CKT by Murdoch University Australian Postgraduate Award and was supported by funding from the Western Australian Government's State NRM Program, to SSG by a Discovery Early Career Researcher Award (Australian Research Council). As a component of the collaborative, 'Woylie Conservation Research Project', lead by DEC, funding was also provided by DEC ('Saving Our Species' initiative), WA State NRM program and Caring for Our Country federal funds. Trapping of woylies at UWR was conducted by DEC, and at KWS by AWC and with animal ethics approval from the DEC AEC. The woylies were managed and handled using procedures formally approved by the Murdoch University Animal Ethics Committee (AEC number: W2350-10) and Department of Environment and Conservation Animal Ethics Committee (AEC number: DEC 36/2010) in compliance with the Australian Code of Practice for the use of Animals for Scientific Purposes. In accordance with section 8.5 of the ICZN's International Code of Zoological Nomenclature, details of the new species have been submitted to ZooBank with the life science identifier (LSID) zoobank.org:pub:3DA85A31-0B18-4BF6-A82D-079286D89A3F.

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Received: 26 February 2013 Accepted: 22 April 2013
Published: 26 April 2013

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doi:10.1186/1756-3305-6-121
Cite this article as: Thompson *et al.*: Morphological polymorphism of *Trypanosoma copemani* and description of the genetically diverse *T. vegrandis* sp. nov. from the critically endangered Australian potoroid, the brush-tailed bettong (*Bettongia penicillata* (Gray, 1837)). *Parasites & Vectors* 2013 **6**:121.

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