A high prevalence of *Theileria penicillata* in woylies (*Bettongia penicillata*)

Jia Rong[a], Michael Bunce[b], Adrian Wayne[c], Carlo Pacioni[a], Una Ryan[a], Peter Irwin[a,]

[a] School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia

[b] School of Biological Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia

[c] Science Division, Department of Environment and Conservation, Manjimup, Western Australia 6258, Australia

Abstract

The woylie or brush-tailed bettong (*Bettongia penicillata*) is a medium-sized native Australian marsupial that has undergone a dramatic decline in numbers in recent years. Trypanosome parasites have been identified in the woylie but little is known about the prevalence and clinical impact of other haemoprotozoan parasites in these marsupials. In the present study, the occurrence and molecular phylogeny of a piroplasm was studied in woylies from six different sites in Western Australia (WA). Blood samples were screened by PCR at the 18S rRNA locus and 80.4% (123/153) of the blood samples were positive for piroplasm DNA. Sequence and phylogenetic analysis of 12 of these positives identified them as *Theileria penicillata*, and sequencing of cloned PCR products indicated that no other species of *Theileria* were present. Infected woylies had a lower body weight but microscopic evaluation of the blood films indicated that *T. penicillata* did not appear to cause red cell injury or anaemia. Further studies are required to determine the clinical significance of *T. penicillata* in woylies.

Keywords: Piroplasms; Brush-tailed bettong; Wildlife; Marsupial; *Theileria*; Phylogeny

1. Introduction

The woylie or brush-tailed bettong (*Bettongia penicillata*) is a medium-sized marsupial belonging to the Potoroidae family that once inhabited more than 60% of the Australian mainland but now survives in only a few locations in Western Australia, and in translocated populations in South Australia and New South Wales (Groom, 2010). Although changes in land use and predation by introduced species are generally implicated in the historical reduction in woylie numbers Australia-wide, the most recent declines in the woylie populations have occurred despite no apparent increase in the number or type of predators in the region and no apparent decrease in natural resource (Wayne, 2008). The woylie is currently listed as a critically endangered marsupial species on the IUCN Red List of Threatened Species (Wayne et al., 2008a).

Potential causes for the woylie decline include introduced predators such as the European red fox and feral cat (Christensen, 1980 and Start et al., 1995), and parasitic or infectious diseases. *Toxoplasma* and *Trypanosoma* parasites were recently reported to be common in the woylie population (Wayne, 2008, Smith et al., 2008, Averis et al., 2009 and Paparini et al., 2011).

Theileria and *Babesia* are haemoprotozoa transmitted by arthropod vectors. To date there has been no systematic study of native Australian haemoprotozoa and relatively little is understood of their epidemiology and impact. However, species within both genera cause significant economic impacts in domesticated animals worldwide, resulting in major losses of cattle and other production animals (Heim et al., 2007). Although little is known about the clinical effects of piroplasm infections in marsupials, high parasitaemias appear to be well tolerated in some species (e.g. the Gilbert's potoroo, *Potorous gilbertii*) and have little pathological effect (Lee et al., 2009), yet in others, such as the agile antechinus (*Antechinus agilis*), babesiosis has been reported as a cause of anaemia in moribund males undergoing semelparity (Cheal et al., 1976 and Barker et al., 1978).

Currently there are seven named *Theileria* spp. in native Australian mammals: *Theileria tachyglossi* in the echidna (*Tachyglossus aculeatus*) (Priestley, 1915); *Theileria peramelis* in the northern brown bandicoot (*Isoodon macrourus*) (originally reported as *Isoodon obesulus*), the long nosed bandicoot (*Perameles nasuta*) and the long nosed potoroo (*Potorous tridactylus*), *Theileria ornithorhynchi* in the platypus (*Ornithorhynchus anatinus*) (Mackerras, 1959); *Theileria gilberti* in Gilbert's potoroo (*P. gilbertii*) (Lee et al., 2009); *Theileria brachyuri* in the quokka (*Setonix brachyurus*), *Theileria fuliginosa* in the western grey kangaroo (*Macropus fuliginosus*), and *Theileria penicillata* in the woylie (*B. penicillata*) (Clark and Spencer, 2007). Unnamed piroplasm species (presumed *Theileria* spp.) have been found in the long-nosed bandicoot (*P. nasuta*) and long-nosed potoroo (*P. tridactylus*) (Clark et al., 2004 and Lee et al., 2009), and, recently, in the burrowing bettong (boodie) (*Bettongia lesueur*) (Paparini et al., 2012). There are at least two named *Babesia* spp. in Australian marsupials and monotremes: *Babesia tachyglossi* in the echidna (*T. aculeatus*) (Backhouse and Bolliger, 1959), *Babesia thylacis* in the northern brown bandicoot (*I. macrourus*) (originally reported as *I. obesulus*) (Mackerras, 1959) and the northern quoll (*Dasyurus hallucatus*) (Bangs and Purnomo, 1996); plus a number of un-named piroplasms (presumed *Babesia* spp.) in the agile antechinus (*A. agilis*), echidna (*T. aculeatus*), Proserpine rock-wallaby (*Petrogale persephone*), and woylie (*B. penicillata*) (Arundel et al., 1977, Barker et al., 1978, Clark et al., 2004 and Paparini et al., 2012). In addition, several *Babesia* spp. have been reported in South American marsupial opossums (*Didelphis marsupialis* and *Didelphis*

albiventris) (Da Serra Freire, 1979 and Herrera and Urdaneta-Morales, 1991). The aim of the present study was to determine the prevalence of piroplasms in woylies, to identify the species present and to determine any clinical impact they may have on this Western Australian marsupial.

2. Materials and methods

2.1. Sampling

Trapping was conducted as a component of the Woylie Conservation Research Project (Wayne, 2008), a multidisciplinary research program including a population comparison study investigating the health and decline of the woylie in Western Australia (WA) coordinated by the management committee of the Woylie Disease Reference Council (WDRC). Between 2006 and 2008 woylies were trapped at three locations in the south of WA including Karakamia, Kingston (Warrup, Winnejup and Corbal forest sites) and Perup (Keninup and Boyicup forest sites) (Table 1). Biological data such as the animals' sex, weight, reproductive status and clinical score were assessed and a total of 153 blood samples were collected from the lateral tail vein into EDTA tubes and stored at 4 °C. Upon return from the field, blood samples were submitted to Murdoch University Clinical Pathology Laboratory and processed within 36 h of collection, as recommended for macropodid blood samples by Hulme-Moir et al. (2006). Haematological parameters were established with an automatic haematology analyser (ADVIA-120) using multi-species software (Bayer Animal Health Diagnostics Division, Tarrytown, NY, USA) including; total white blood cell counts (WBC, $\times 10^9$/L), red blood cell counts (RBC, $\times 10^{12}$/L) and platelet counts (PLT, $\times 10^9$/L).

Table 1. Prevalence of *Theileria* in woylies at the various sites analyzed. 95% confidence intervals are given in parentheses.

Localities	Trapping site	Microscopy positives	PCR positives	Prevalence (%)
Perup	Keninup	24/24	35/35	100 (100–100)
	Boyicup	Not done	5/5	100 (100–100)
Kingston	Corbal	Not done	20/20	100 (100–100)
	Winnejup	Not done	14/19	73.7 (53.9–93.5)
	Warrup	0/4	6/30	20 (5.7–34.3)
Karakamia	Karakamia	4/4	43/44	97.7 (93.3–102.1)
Total		28/32	123/153	80.4 (74.1–86.7)

2.2. Microscopy

Blood smears were made from 24 samples from Keninup, four samples from Warrup and four samples from Karakamia, and were stained with a modified Wright-Giemsa stain using an Ames Hematek slide stainer (Bayer, Germany). Blood films were examined under light microscopy at magnifications of 400× and 1000× (oil immersion) using a BX50 microscope (Olympus, Japan) to identify erythrocytic inclusions. The morphology of parasites and red blood cells was noted, followed by an estimate of average parasitaemia using a standard technique (Callow, 1984).

2.3. DNA extraction and PCR

DNA was isolated from 100 μL of blood from each sample (n = 153) using the MasterPure Purification Kit (Epicentre Biotechnologies, USA) according to the manufacturer's instructions. A fragment of piroplasm DNA was amplified using nested primers to the 18S rRNA gene as previously described (Jefferies et al., 2007). For all PCRs, strict contamination controls were applied: PCRs were set-up in UV-irradiated, DNA free rooms with separate pipettes and aerosol-resistant tips. All PCR reagents were stored in the DNA-free room and were not in contact with DNA. Negative controls (no DNA) were included in every run. PCR products were visualized on a 1% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA), and imaged with a dark reader trans-illuminator (Clare Chemical Research, USA). Amplicons corresponding to the expected length were excised, purified using a MO BIO UltraClean DNA purification kit (MOBIO Laboratories, USA), and sequenced using an ABI Prism BigDye Terminator Cycle Sequencing kit (v3.1 Applied Biosystems, USA), on an 3730 genetic Analyser (Applied Biosystems).

2.4. Cloning

To determine if multiple species of piroplasms were present in the blood, four separate 18S nested PCR products were cloned in the pGEM-T Easy Vector System II (Promega, USA). After transformation of *Escherichia coli* JM109 competent cells, plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Germany) from cultured clones grown overnight, and sequenced as described above, or using SP6 and T7 promoter primers (Promega, USA).

2.5. Phylogenetic analysis

Nucleotide sequences were analysed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with all the available sequences of *Theileria* species known to infect marsupials and with common species found in domestic animals from GenBank using ClustalW (http://www.clustalw.genome.jp). jModeltest 0.1.1 (David Posata - http://darwin.uvigo.es/) was used to select an appropriate evolutionary model. Distance estimation was conducted using TREECON (Van de Peer and De Wachter, 1994), based on evolutionary distances calculated with the Tamura–Nei model and grouped using Neighbor-Joining. Parsimony analyses were conducted using MEGA version 4 (Tamura et al., 2007). One thousand Bootstrap replicates were conducted to assess the stability of tree topologies. Maximum Likelihood (ML) analyses were conducted using the program PhyML (Dereeper et al., 2008) and the reliability of the inferred trees was assessed by the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006).

2.6. Statistical analysis

Statistical difference of biological data (body weight, RBC, WBC and platelet count) between positive and negative individuals, across the three localities (Karakamia, Kingston and Perup) were investigated using two-way ANOVA and Chi-square test was used to evaluate differences in the prevalence of piroplasms between locations using SPSS® software (SPSS v19 SPSS, Chicago, IL). To avoid the confounding effect of age, sexual dimorphism and presence of small pouch young (which could not be removed from the females for welfare reasons), analysis involving body weight was limited to only adult males.

3. Results

3.1. Haematology

Small intra-erythrocytic ovoid and pyriform-shaped parasites, similar to those described by Clark and Spencer (2007) were detected by microscopy in all 24 samples (100%) examined from Keninup, in 4/4 of the samples from Karakamia, but in none of the four samples sourced from Warrup (Fig. 1). Normal red cell density in the blood smear and unremarkable erythrocyte morphology was noted in all cases, with no signs of damaged or immature cells and anaemia was not detected in any individuals. Haematological indices are reported in Table 2.

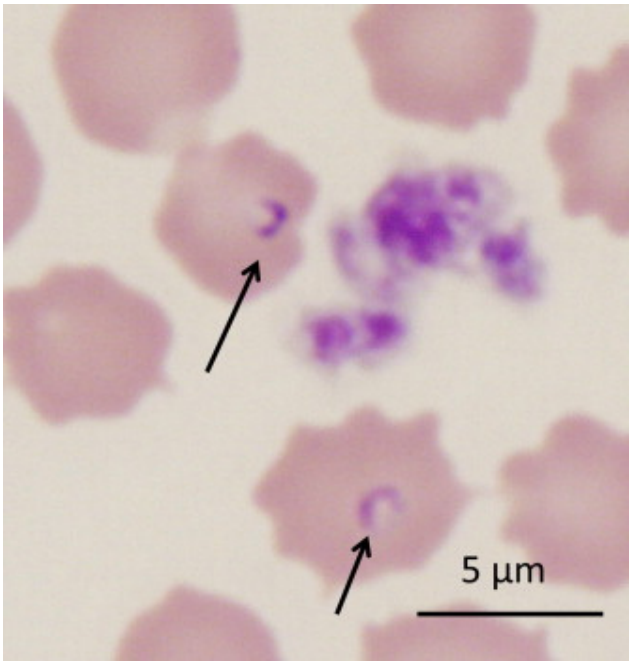

Fig. 1. Photomicrograph of a blood film from a woylie isolate (Woylie 1) (Giemsa stain, original magnification 1000×) showing intra-erythrocytic ring-like structures similar to the morphology described for *Theileria penicillata* parasites described by Clark and Spencer (2007), together with a group of platelets.

3.2. Nested PCR screening and sequence analysis

The overall prevalence of piroplasm DNA in the samples was 80.4% (123/153) (Table 1). The prevalence in the different sites ranged from 20% in Warrup to 100% in Keninup, Boyicup and Corbal. Sequences were obtained for 12 of these (uncloned) positives. Sequence alignments indicated that all these sequences were 100% identical to *T. penicillata* previously described in woylies by Clark and Spencer (2007). Sequences were also obtained for 24 clones, all of which were *T. penicillata* i.e. no mixed infections were identified. Phylogenetic analysis using distance and parsimony produced consistent tree topologies and revealed that the *T. penicillata* sequences from the woylie isolates from the present study were closely related (0.2% genetic distance) to a *Theileria* species isolated from the Long Nosed Potoroo (Fig. 2-NJ tree shown). Two GenBank sequences were obtained for *T. brachyuri*: isolate PSC5 (DQ437684) and isolate PSC12 (DQ437685). However, it appears that these sequences from the same host are genetically distinct as isolate PSC5 grouped most closely with *T. gilberti* while isolate PSC12 grouped most closely with *T. fuliginosa*. The two *T. brachyuri*

isolates were 3.4% divergent from each other, which strongly suggest that they are in fact separate species.

Table 2. Mean body weight, white blood cell (WBCC) count, red blood cell count (RBCC), and platelet and standard error of infected and uninfected woylies.

Infected/Uninfected	*Locality*	*Body weight (g)*[a]	*WBCC × 109 /L*	*RBCC × 1012 /L*	*Platelets × 109 /L*
PCR negative	*Perup*	–	–	–	–
	Kingston	*1388.3 ± 22.3*	*4.7 ± 0.5*	*10.3 ± 0.4*	*604.1 ± 80.8*
	Karakamia	*1100*[b]	*4.4 ± 2.5*	*10.1 ± 1.8*	*429 ± 379.1*
	Total	*1370.3 ± 27.6*	*4.6 ± 0.4*	*10.3 ± 0.2*	*596.5 ± 32.9*
PCR positive	*Perup*	*1369.3 ± 31.9*	*7 ± 0.5*	*11.7 ± 0.4*	*473.4 ± 75.8*
	Kingston	*1386.6 ± 25.1*	*6.8 ± 0.5*	*10.6 ± 0.4*	*632.1 ± 75.8*
	Karakamia	*1058.1 ± 24.1*	*3.4 ± 0.6*	*10 ± 0.4*	*520.1 ± 84.8*
	Total	*1300.2 ± 23.5*	*5.9 ± 0.4*	*10.8 ± 0.2*	*543.4 ± 50.8*

[a] Adult males only.
[b] No SE was calculated for this locality as only one male was negative.

3.3. Statistical analysis

Biological (weight) and haematological data in infected and uninfected individuals were evaluated for the effect of *Theileria* infection (Table 2). While infected animals had a lower mean body weight than uninfected individuals, the difference was not significant when taking into account the variability between localities, which in itself had a significant effect ($p < 0.0005$). Similarly, a mean difference for RBC and platelet counts was found between localities ($p = 0.005$ and $p = 0.008$, respectively) with no significant effect of the infective status.

A significant difference in prevalence of piroplasm infection was found between localities, with Kingston having a lower prevalence than the other two localities (Chi-square = 40.15, df = 2, $p < 0.0005$).

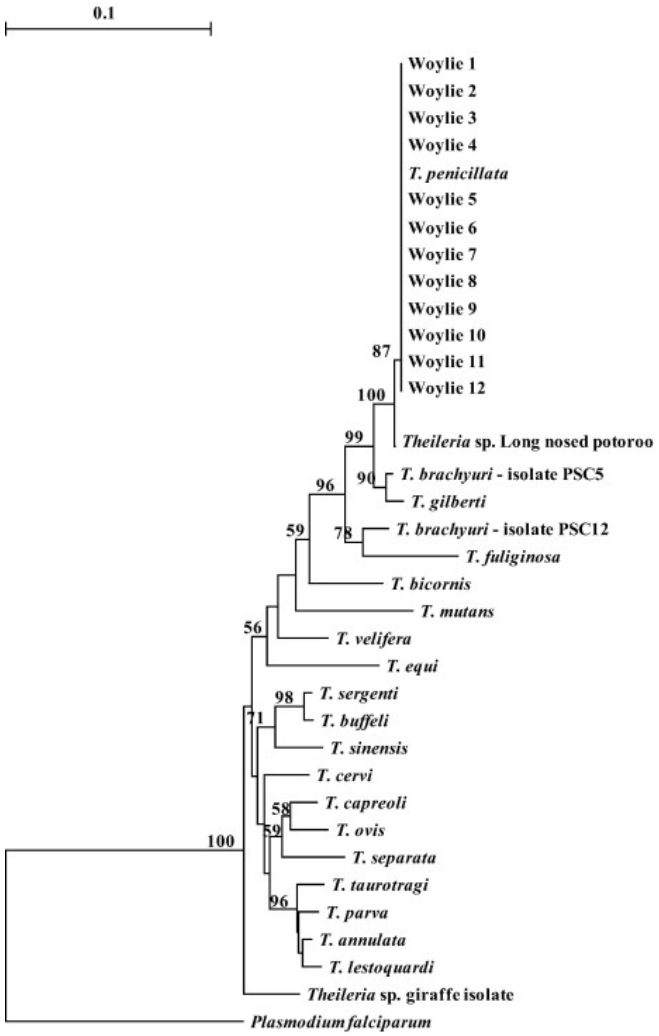

Fig. 2. Phylogenetic analysis of the relationships of *Theileria* species including woylie-derived isolates, based on 18S rDNA partial sequences. Evolutionary history was inferred using Neighbor-Joining analysis. Percentage support (>50%) from 1000 pseudo-replicates is indicated at the left of the supported node. Woylie isolates 1–6 were from Keninup, 7–10 from Karakamia and woylie isolates 11–12 were from Corbal (see Table 1).

4. Discussion

In the present study, a high prevalence (80.4%) of *T. penicillata* was detected in woylies from three locations (six forest trapping sites) in Western Australia. This is the first study to determine the prevalence of *Theileria* in woylies; a previous publication that identified and named *T. penicillata* examined blood from only a single individual (Clark and Spencer, 2007). All the sites tested in this study had high numbers of parasitaemic woylies (73–100%) with the exception of Warrup, a trapping site in the Kingston locality where the prevalence was much lower (20%). Interestingly, the woylie population at this site, having declined more than 80% in 1999–2001 to support relatively low numbers of woylies in 2001–2004, is so far the only site in WA to have undergone a significant recovery to moderate densities (2005–2008), although since 2009 it has declined again by around 90% (Wayne et al., 2008b). Given that there are no known significant environmental differences between Warrup and the other sites in Kingston and Perup (they are geographically close and have similar ecology), the low parasitaemia in the Warrup woylies may reflect a change in the subtle balance in the host-parasite-vector relationships associated with woylie declines. It is possible, for example, that

a reduction in vector concentration accompanied the decline in the host population, resulting in lower parasite transmission rates once the recovery in woylie numbers had started. Alternatively, the recovering population of woylies may possess a genetic advantage and be immunologically more competent at controlling piroplasm numbers.

Despite being recognized for nearly a century, relatively little information is available on infection dynamics of *Theileria* spp. in native hosts. Previous reports suggest that the prevalence of *Theileria* spp. in Australian monotreme and marsupial hosts is relatively high; Collins et al. (1986) found 53/54 (98%) platypuses trapped in New South Wales were infected with *T. tachyglossi* and a similar prevalence, 16/16 (100%), was reported for *T. gilberti* in Gilbert's potoroos in Western Australia (Lee et al., 2009). In our study, like that of Lee et al. (2009), we also utilized PCR analysis to detect piroplasm infection rates; this method confirmed the high prevalence of infection in all but the Warrup site (Table 1) and although only a subset of samples were screened by microscopy (32/153), all the microscopy positives corresponded with the PCR positives. The results of the present study suggest that the parasite is widespread among woylies and can probably be considered to be endemic in this host species in these localities in Western Australia. A recent study, which examined the prevalence of trypanosomes in various native marsupials, identified mixed trypanosome species in woylies from a different geographical locality (Paparini et al., 2011). However, in the present study, sequence analysis of cloned PCR products indicated that no mixed infections were present.

Analysis of *T. penicillata* 18S data revealed a close phylogenetic relationship with a *Theileria* sp. from the Long Nosed Potoroo (*P. tridactylus*) (specimens collected in Victoria; Lee et al., 2009) which is not surprising given the host species' close evolutionary history (Westerman et al., 2004).

The clinical impact of *Theileria* on the woylies is unclear. Infected woylies had a lower body weight but microscopic evaluation of the blood smears revealed that the blood cell morphology was normal, which together with the normal red cell count indicates that *T. penicillata* does not cause red cell injury or anaemia, at least in the individuals for which blood films were examined. Indeed, the difference in body weight between infected and uninfected woylies may have been caused by factors that were not investigated in this study (e.g. adaptation of the Karakamia population to high density, Wayne, 2008). Overall our study suggests that *T. penicillata* may not be especially pathogenic in woylies, as also seems to be the situation in Gilbert's potoroos infected with *T. gilbertii* (Lee et al., 2009), however further research involving larger numbers of animals and, ideally, woylies that are clinically unwell, is required to confirm this. An expansion of this study into a more comprehensive longitudinal and comparative investigation of the prevalence and parasitaemia levels of *T. penicillata* in woylies across different locations at different stages of the decline would also determine the strength of the possible association between the parasite and the host's spectacular decline.

References

Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. Systematic Biology 55, 539–552.

Arundel, J.H., Barker, I.K., Beveridge, I., 1977. Diseases of marsupials. In: Stonehouse,vB., Gilmore, D. (Eds.), The Biology of Marsupials. Macmillan Press, Sydney, pp. 141–154.

Averis, S., Thompson, R.C.A., Lymbery, A.J., Wayne, A.F., Morris, K.D., Smith, A., 2009. The diversity, distribution and host-parasite associations of trypanosomes in Western Australian wildlife. Parasitology 136, 1269–1279.

Backhouse, T.C., Bolliger, A., 1959. Babesia tachyglossi n. sp. from the echidna *Tachyglossus aculeatus*. Journal of Protozoology 6, 320–322.

Bangs, M.J., Purnomo, 1996. *Babesia thylacis* (Apicomplexa: Babesiidae) in a northern quoll, *Dasyurus hallucatus* (Marsupialia: Dasyuridae), from Western Australia. Journal of the Helminthological Society of Washington 63, 266–268.

Barker, I.K., Beveridge, I., Bradley, A.J., Lee, A.K., 1978. Observations on spontaneous stress-related mortality among males of the dasyurid marsupial *Antechinus stuartii* Macleay. Australian Journal of Zoology 26, 435–447.

Callow, L.L., 1984. Bovine babesiosis in Australia. In: Callow, L.L. (Ed.), Animal Health in Australia. Protozoal and Rickettsial Diseases, AGPS, Canberra, p. 148.

Cheal, P.O., Lee, A.K., Barnett, J.L., 1976. Changes in the haematology of *Antechinus stuartii* (Marsupialia) and their association with male mortality. Australian Journal of Zoology 24, 299–311.

Clark, P., Adlard, R.D., Spratt, D.M., 2004. Haemoparasites of Australian mammals. In: Clark, P. (Ed.), Haematology of Australian Mammals. CSIRO Publishing, Collingwood, pp. 147–151.

Clark, P., Spencer, P.B.S., 2007. Description of three new species of *Theileria* Bettencourt, Franca & Borges, 1907 from Macropodoidea in Western Australia. Transaction of the Royal Society of South Australia 131, 100–106.

Collins, G.H., Whittington, R.J., Canfield, P.J., 1986. *Theileria ornithorhynchi* Mackerras, 1959 in the platypus, *Ornithorhynchus anatinus* . Journal of Wildlife Diseases 22, 19–24.

Christensen, P.E.S., 1980. A sad day for native fauna. Forest Focus 23, 3–12.

Da Serra Freire, N.M., 1979. *Babesia ernestoi* n.sp. in *Didelphis marsupialis* L., 1758, and *D. albiventris* Lund, 1841, in Brazil. Zentralblatt fur Veterinarmedizin Reihe B 26, 614–622.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O., 2008. Phylogeny: robust phylogenetic analysis for the non-specialist. Nucleic Acids Research 36, W465–469.

Groom, C., 2010. Justification for continued conservation efforts following the delisting of a threatened species: a case study of the woylie, *Bettongia penicillata ogilbyi* (Marsupialia: Potoroidae). Wildlife Research 37, 183–193.

Heim, A., Passos, L.M.F., Ribeiro, M.F.B., Coasta-Junior, L.M., Bastos, C.V., Cabral, D.D., Hirzmann, J., Pfister, K., 2007. Detection and molecular characterization of *Babesia caballi* and *Theileria equi* isolates from endemic areas of Brazil. Parasitology Research 102, 63–68.

Herrera, L., Urdaneta-Morales, S., 1991. *Didelphis marsupialis* : reservoir of *Babesia brasiliensis* in the valley of Caracas (Venezuela). Acta Cientifica Venezolana 42, 45–46.

Hulme-Moir, K.P., Clark, P., Spencer, P., 2006. Effects of temperature and duration of sample storage on the haematological characteristics of western grey kangaroos (*Macropus fuliginosus*). Australian Veterinary Journal 84, 143–147.

Jefferies, R., Ryan, U.M., Irwin, P.J., 2007. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. Veterinary Parasitology 144, 20–27.

Lee, J.Y., Ryan, U.M., Jefferies, R., McInnes, L.M., Forshaw, D., Friend, J.A., Irwin, P.J., 2009. *Theileria Gilberti* n. sp. (Apicomplexa: Theileriidae) in the Gilbert's potoroo (Potorous gilbertii). Journal of Eukaryote Microbiology 56, 290–295.

Mackerras, M.J., 1959. The haematozoa of Australian mammals. Australian Journal of Zoology 7, 105–135.

Paparini, A., Irwin, P.J., Warren, K., McInnes, L.M., de Tores, P., Ryan, U.M., 2011. Identification of novel trypanosome genotypes in native Australian marsupials. Veterinary Parasitology 183, 21–30.

Paparini, A., Ryan, U.M., Warren, K., McInnes, L.M., de Tores, P., Irwin, P.J., 2012. Identification of novel *Babesia* and *Theileria* genotypes in the endangered marsupials, the woylie (*Bettongia penicillata*) and boodie (*Bettongia lesueur*). Experimental Parasitology, accepted for publication.

Priestley, H., 1915. *Theileria tachyglossi* (n. sp.) A blood parasite of *Tachyglossus aculeatus* . Annals of Tropical Medicine and Parasitology 9, 233–238.

Smith, A., Clark, P., Averis, S., Lymbery, A.J., Wayne, A.F., Morris, K.D., Thompson, R.C., 2008. Trypanosomes in a declining species of threatened Australian marsupial, the brush-tailed bettong *Bettongia penicillata* (Marsupialia: Potoroidae). Parasitology 135, 1329–1335.

Start, T., Burbidge, A., Armstrong, D., 1995. Woylie recovery plan. Department of Conservation and Land Management, Western Australia. Wildlife management. Program 16, 19.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596–1599.

Van de Peer, Y., De Wachter, R., 1994. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Computer Applications in the Biosciences 10, 569–570.

Wayne, A.F. (Ed.), 2008. Diagnosis of Recent Woylie (*Bettongia penicillataogilbyi*) Declines in southwestern Australia. In: Progress report of the Woylie Conservation Research Project. Western Australian Government Department of Environment and Conservation, Perth, Western Australia.

Wayne, A. F., Friend, T., Burbidge, A., Morris, K. van Weenen, J., 2008a. *Bettongia penicillata* . In: IUCN Red List of Threatened Species. www.iucnredlist.org.

Wayne, J.C., Wayne, A.F. Wilson, I., 2008b. Upper Warren fauna monitoring. In: Wayne, 2008 , pp. 32-42.

Westerman, M., Loke, S., Springer, M.S., 2004. Molecular phylogenetic relationships of two extinct potoroid marsupials, Potorous platyops and caloprymnus campestris (Potoroinae: Marsupialia). Molecular Phylogenetics and Evolution 31, 476–485