Prevalence and Intensity of Trypanosome Infections in Stable and Declining Populations of Brush-Tailed Bettongs (Bettongia Penicillata)

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Prevalence and intensity of trypanosome infections in stable and declining populations of brush-tailed bettongs (*Bettongia penicillata*)

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Abstract
The brush-tailed bettong (*Bettongia penicillata*), or woylie, is an endemic Australian marsupial that has recently suffered a large population crash in the Upper Warren region of Western Australia. Research has shown that both the declining Upper Warren population of brush-tailed bettongs and a stable population at Karakamia Wildlife Sanctuary are infected with a novel *Trypanosoma* sp., and the declining Upper Warren population was found to have a higher prevalence of trypanosome infection than that of the stable Karakamia population. In this study, further work was done to see if 1) the prevalence and intensity of trypanosome infection still differed significantly between the two populations, 2) if the prevalence and intensity were indicative of the overall health of the population, and 3) the infection rate varied seasonally. Using light microscopy, no blood samples taken from Karakamia were found to show evidence of trypanosome infection, while an average of 23.5% and 28.6% of samples taken from Keninup in May 2009 and October 2009, respectively, showed evidence of trypanosome infection. Individual parasitemia levels in those infected slides were extremely low, and no seasonal variation in prevalence or intensity could be found. While there are significant differences in trypanosome prevalence (and therefore intensity) between the two populations, this does not mean that trypanosomes are the ultimate cause of the population crash; co-infection with other protozoan parasites or condition-dependent virulence are both possible alternate causes of the crash. Further research and analysis will help to formulate successful conservation policies that can facilitate the recovery of the critically endangered woylie populations.

**Keywords:** *Bettongia penicillata*, woylie, trypanosome, population decline, parasite
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1. Introduction

1.1 Conservation and Disease

Human expansion and development across the world has dramatically affected wild ecosystems and has jeopardized the survival of several animal and plant species globally. The possible consequences of human use of formerly undisturbed land include habitat destruction, introduction of non-native species, and overhunting. In Australia, the native flora and fauna have been affected greatly by large-scale human development initiated two centuries ago by the arrival of Europeans.

In the past 200 years, 50% of global mammal extinctions have occurred in Australia (Short and Smith, 1994). As of 2009, 1,142 mammal species globally are considered “threatened” (a category that encompasses critically endangered, endangered, and vulnerable species, all of which are considered to be in danger of becoming extinct in the near future), and 57 of those mammal species are located in Australia (IUCN, 2009). The two taxonomic groups that have been most affected by these population declines and extinctions in Australia are rodents and marsupials (Short and Smith, 1994).

Traditionally, wildlife conservationists believed that protection of habitat of endangered species was the most important factor affecting the survival of the species, and only criteria of the land (such as habitat rarity, habitat diversity, or vegetation structure) were evaluated in conservation schemes (Margules and Usher, 1981). Beginning in the 1970s, new ideas about conservation and its practice began to infiltrate the scientific community. One such idea was the application of island biogeography in the planning of conservation reserves; instead of unsuccessfully lobbying for governments to set aside large tracts of land for reserves, systems of reserve “islands” could be set up to protect threatened species. Disease was fleetingly addressed in these studies, but the size and habitat features of “island” reserves were still considered more important for the survival of a species than the habitat’s potential for preventing disease transmission (Diamond, 1976).

Then, Anderson and May published papers in 1979 investigating the population biology of infectious diseases, and conservationists began to question more whether disease could be an equally important factor affecting the conservation of endangered species (Anderson and May, 1979). In further studies, it was shown that parasite infection can have serious effects on the survival and reproduction of host species, and that both habitat and
disease issues must be addressed to design effective reserves that maintain the overall health and genetic diversity of a species (Scott, 1988).

One of the studies that implicated disease as a contributing factor to the extinction of a wild species investigated the extinction of the black-footed ferret (*Mustela nigripes*) in Wyoming. The black-footed ferret had been presumed extinct in the 1979 after the last ferret in captivity died, but in 1981, one colony was discovered in Meeteetse, Wyoming. Although all efforts were made to minimize the ferrets’ exposure to disease from researchers, in 1985, a canine distemper epizootic (most likely originating from a domesticated dog) broke out within the single colony. Government scientists managed to capture eighteen ferrets that were not infected with the disease, and the scientists hoped that captive breeding would save the species (Thorne and Williams, 1988). Today, there are 750 individuals in the wild and 250 in captivity, and scientists credit vigilant disease monitoring along with habitat restoration and breeding programs as the reasons for the black-footed ferrets’ successful rebound from the edge of extinction.

Disease has been implicated in the decline or extinction of other species; some examples include the population declines of anurans in Australia and Panama in the 1990s by infection with a chytridiomycete fungus (Berger et al., 1998), the drastic decline of wild ungulates in Sub-Saharan Africa in the 1890s due to morbillivirus rinderpest infection (McCallum and Dobson, 1995), and possibly the extinction of the Australian thylacine in the early 20th century by canine distemper (Guiler, 1961). Disease has the potential to decimate a species, and thus it is of special concern in Australia where so many endemic species are threatened or endangered.

1.2 Disease and the Woylie

The brush-tailed bettong (*Bettongia penicillata*), or woylie, is one of Australia’s endangered species. An endemic marsupial, the species was once widely dispersed across the southern regions of Australia, but habitat destruction coupled with predation by introduced foxes and feral cats precipitated a population crash in the 1950s and 1960s. By the 1970s, only three populations existed in the wild (Wayne et al., 2009). As a result of conservation and fox-baiting efforts, the wild woylie populations grew, and by 1996 the woylie was removed from the State and Commonwealth threatened species list (DEC, 2008).
In spite of these successful conservation efforts, beginning in 2001, wild populations of woylies began to decline again. According to the Department of Environment and Conservation, in the past 5 years, the wild woylie population has dropped to 10,000 individuals, a 75% decrease in numbers (DEC, 2008). In contrast, those woylie populations that were in protected sanctuaries remained stable in the same time period. The population decrease of wild woylies has led to a re-listing of the species on the State and Commonwealth threatened list and inquiries into what has caused this population crash.

Initial hypotheses questioned whether a resource shortage or increased predation had led to the decline. The DEC looked into both of these possible causes but found no evidence for either (DEC, 2008). Scientists also noted that the source of the decline was increased adult mortality, not emigration of woylies out of the area or decreased birth rates. Based on these findings, it became clear that disease was a factor worth evaluating as a possible cause of the population crash.

A study soon showed that a novel *Trypanosoma* sp. was found in both declining and stable populations of woylies (Smith et al., 2009). This study also found that the prevalence of the trypanosome was higher in the declining population as compared to the stable population (Smith et al., 2009). These discoveries indicate that infection by trypanosomes could be one element leading to a decline in population.

1.3 Justification of Study

In 2009, Smith et al. completed a comparison study of two woylie populations, examining the prevalence of trypanosomes in a stable population at Karakamia Wildlife Sanctuary and a declining population in the Upper Warren Region. Further data needs to be collected to determine whether infection rates are changing or remaining constant in these two populations and whether infection rates are truly indicative of their overall health. Also, Smith et al. did not address whether there is seasonal variation in trypanosome infection prevalence in these two populations. Measuring seasonality could help to determine what ectoparasite is possibly transmitting the trypanosomes to woylies and other mammals, as most ectoparasites have characteristic seasonal population fluctuations (Heath, 1978).

1.4 Aims of Study

The aims of this study were to determine not only the prevalence of trypanosomes in the Karakamia and Upper Warren populations, but also the intensity of the infections by
quantifying the number of trypanosomes seen in blood samples. By measuring both the prevalence and intensity, a better understanding of how trypanosomes are affecting the woylies on both a population-wide level and an individual level could be gained. Also, this study seeks to determine whether there are any seasonal changes in trypanosome prevalence and intensity in either of the two populations.

2. Methods

2.1 Study Sites

Blood samples were collected from two sites. The first site, Karakamia Wildlife Sanctuary, is located 50 km northeast of Perth (S31.03038; E116.24604) and covers 275 hectares of Jarrah forest ecosystems (Figure 1). At least 15 Australian endangered species live within the sanctuary, including the woylie, numbat, and tammar wallaby. The sanctuary is fenced to prevent predators such as feral dogs and cats from entering the property and possibly killing wildlife. The Australian Wildlife Conservancy, a non-governmental organization, runs the sanctuary.

The second site, Keninup Creek, is located within Perup Nature Reserve (S34.11528; E116.32362) in the Upper Warren catchment, which lies approximately 300 km southeast of Perth and covers an area of 300,000 hectares (Figure 1). The nature reserve is unfenced.

2.2 Study Organisms

Brush-tailed bettongs (Bettongia penicillata) (Figure 2), or woylies, are small marsupials endemic to Australia. They are a largely nocturnal species, resting during the day in nests constructed with leaves and bark and coming out at night to feed on fungi and tubers. This feeding behavior is an important ecological contribution of the woylie; in digging underground for tubers and fungi, the woylies create holes that allow water to seep into the ground and move nutrients there more easily. They are also important seed dispersers for fungi and plants.

Trypanosomes are protozoan parasites in the genus Trypanosoma that are known to infect woylies along with many other animals and insects. The parasites themselves are unicellular with a single flagellum and a kinetoplast where the majority of their DNA is stored in a large mitochondrion (Figure 3). They primarily live in insect hosts, though some
species of *Trypanosoma* have a life cycle requiring the infection of a secondary host for reproduction and dispersal. These species, perhaps most well known as the causative agents of African sleeping sickness (*T. brucei*) and Chagas’ disease (*T. cruzi*), have evolved to infect a certain range of species. There are several species of *Trypanosoma* that specifically infect Australian marsupials (Averis et al. 2009).

**Figure 1.** Map showing woylie sampling sites at Karakamia Wildlife Sanctuary and the Upper Warren Region. Large black dot within the Upper Warren Catchment box shows location of Keninup Creek. Courtesy of the Woylie Conservation Research Project and the DEC.
Figure 2. Woylie. Courtesy of the DEC.

Figure 3. Diagram of a trypanosome. Courtesy of the International Livestock Research Institute.

2.3 Data Collection

2.3.1 Blood collection, blood smear and culture preparation

Blood samples were collected from the woylie population in Karakamia Wildlife Sanctuary on 5 November, 2009. Traps were set up the night before data collection and were baited with a peanut butter, rolled oat, and tinned sardine mixture. The morning of collection (from approximately 6 am to 7 am), woylies were removed from the traps and put into cloth bags. The woylies were then moved to a secure and temperature-controlled shed where blood was taken via syringe from the lateral caudal vein and put directly into a culture tube and a
vial with crystal EDTA (to preserve cytology). A blood smear (one per animal) was then made on microscope slides and left to dry (see Figure 3 for diagram). The smears were later stained with Wright’s and Giemsa stains, and cover slips were applied as well. Blood cultures were left to grow for three weeks; if the woylies from Karakamia had low prevalence levels of trypanosomes, a blood culture would provide an environment where trypanosomes could easily replicate and thus be easily found under the microscope.

For seasonal comparisons, no blood smears from Karakamia from past months were available, but data from a September 2009 microscopic study of blood smears from Karakamia done by Dr. Susana Averis was used (Averis, unpublished data).

Blood samples and smears (one per animal) from Keninup were collected and prepared similarly by DEC officials from 27 to 30 October, 2009, and mailed to Dr. Andrew Smith at Murdoch University. Blood smears collected and prepared by DEC officials in Keninup from 5 to 8 May, 2009 were examined as a seasonal comparison (Table 1).

<table>
<thead>
<tr>
<th>Site</th>
<th>Samples collected</th>
</tr>
</thead>
</table>
| Karakamia | • 28 blood smears from October 2009  
|          | • 26 blood cultures from October 2009  
|          | • Data from 19 blood smears collected and examined in September 2009 by Dr. Susana Averis |
| Keninup | • 17 blood smears from May 2009  
|          | • 21 blood smears from October 2009 |

Table 1. A summary of the samples collected and data used for this study.

**2.3.2 Blood smear and culture analysis**

Blood smear slides and blood cultures were examined for trypanosomes using light microscopy. Slides were examined longitudinally, from the thick section of the smear, through the uniform monolayer of red blood cells, until the “feather” of the smear (Figure 4). For each slide, 200 high power views were examined at a magnification of 400x (10x eyepiece multiplied by a 40x objective lens) and the total number of trypanosomes were counted and recorded. The 200 views were divided evenly between each area (the thick layer, monolayer, and feather), with about 65 views per area. Photographs were taken of some slides using a digital camera and the Canon XP Manager program.
3. Results

In total, 28 blood smear slides (one per animal) from Karakamia and 38 blood smear slides (one per animal) from Keninup were examined. No trypanosomes were observed in any of the 28 Karakamia slides from November 2009. 26 samples from blood cultures (one culture per animal) from Karakamia were also examined three weeks after their collection. None of these 26 blood culture slides had trypanosomes present. Data from Dr. Susana Averis’ examination of blood smears from Karakamia from September 2009 also found that no slides from that time period showed evidence of trypanosomes, either.

The Keninup slides were from two different sampling periods; 17 were from May 5-8, 2009 and 21 slides were from October 27-30, 2009. Of the 17 slides from May 2009, 4 (28.6%) had trypanosomes present, and of the 21 slides from October 2009, 6 (23.5%) slides had trypanosomes present (Figure 5a and 5b, Graph 1).

Trypanosomes were also quantified on slides that showed an infection. The maximum number of trypanosomes found on one slide was 5 (from a Keninup slide from October
2009), and the most common number found was 1 (on 4 slides from both October and May 2009). The average number of trypanosomes found on slides from May 2009 was 2.25, and the average found on slides from October 2009 was 2.33 (Graph 2).

Figure 5. A trypanosome at a) 400x magnification and b) 1000x magnification in a Keninup woylie’s blood smear. Red blood cells are stained red, and the trypanosome is stained purple.

Graph 1. Prevalence of trypanosomes within the Karakamia and Keninup populations at different sampling times.
4. Discussion

4.1 Prevalence and Intensity of Infection

The prevalence of trypanosomes was overall very low, though there were significant differences in prevalence between the Keninup and Karakamia populations. While none of the Karakamia samples, including both blood cultures and blood smears from many different sampling periods, did not have trypanosomes present, approximately 25% of the samples from Keninup from both sampling periods had trypanosomes present.

Similar results were obtained in a previous microscope study of blood smears collected from March 2006 to November 2007 done by Smith et al. in 2009. None of the blood smears obtained from Karakamia woylies showed infection with trypanosomes, while 43% of those blood smears from woylies in the Upper Warren region showed infection (Smith et al., 2009).

This raises an interesting question: are the declining populations declining just because of trypanosome infection? From the data presented in this paper alone, it would seem to be a reasonable hypothesis. Though most trypanosome species are host-specific and non-pathogenic in their hosts, there are exceptions in which trypanosomes cause disease and even death in their hosts. \textit{T. evansi} causes surra in camels, a disease that is the most important cause of mortality and morbidity in camels in North Africa (Enwezor and Sackey, 2005). In
South America and Africa, cattle infected with *T. vivax* develop a hemorrhagic syndrome, and abortions, diarrhea, and death often result (Mwongela et al., 1981).

Other papers, though, have investigated trypanosome infections in wildlife populations and come to different conclusions. First, in their 2009 study of woylie populations, Smith et al. found that there actually were trypanosomes undetectable by microscopy present in the stable Karakamia woylie population using polymerase chain reaction (PCR) methods. If trypanosomes are present in both the stable and declining populations, there must be another factor present in the declining population or its environment that is causing its decline.

One possible factor is contact with predators. Karakamia, site of the stable population, is fenced, while Keninup, site of the declining population, is not; this means woylies at Keninup come into contact with foxes and feral cats, making them susceptible to predation, but also, of equal importance, to possible parasites and diseases that the foxes or cats carry. Feral cats are known reservoirs of *Toxoplasma gondii* (Coman et al., 2008, Ruiz and Frankel, 1980); the *T. gondii* oocyte can infect bettongs and cause acute disease, making them even more vulnerable to predation or secondary infections. *T. gondii* is present in the Keninup and Upper Warren populations of woylies, but not in the Karakamia population (Parameswaran, unpublished data).

Thus, perhaps a dual infection with both *T. gondii* and trypanosomes is causing increased adult mortality of woylies in the Keninup population. Such co-infections with two protozoans commonly occur in nature (Cox, 2001), and it has been shown that in rats, infection with *T. lewisi* is enhanced by co-infection with *T. gondii*, though the mechanism by which this occurs is still unknown (Guerrero et al., 1997).

Another possible cause of increased adult mortality in the Keninup population is the greater stress those woylies experience due to interactions with foxes and feral cats. Bumblebees infected with *Crithidia bombi*, a trypanosome intestinal parasite, had a 50% higher mortality rate if they were put under “stressed” conditions (Brown et al., 2000). It could be that a similar condition-dependent virulence occurs in the Keninup woylie population due to predation by foxes and cats or other stressors.

It is impossible to determine from the data presented in this study whether trypanosomes alone or trypanosomes along with other factors are causing disease in woylie populations. That being said, it seems more likely that a collection of factors is influencing
the health of the woylie populations, as earlier studies have found trypanosomes in both populations and the populations also have differences in *T. gondii* prevalence and amount of contact with predators.

### 4.2 Seasonality

In this study there were no indications that trypanosome prevalence varies with season. For the Karakamia woylies, none of the slides taken in November 2009 or September 2009 had trypanosomes present in the examined blood slides. The non-seasonality of Karakamia trypanosomes is also supported by Smith et al.’s 2009 paper in which no trypanosomes were found on slides taken from June 2006 to November 2007.

In the Keninup woylie population, both prevalence and intensity of infection do not appear to vary with season. There was a 5-percentage point difference between the prevalence rates of May 2009 and October 2009, but the sample sizes in this study were too small for this difference to be statistically significant. The average number of trypanosomes found in slides from May 2009 and October 2009 differed by 3.5%, although again, sample sizes were too small for this to have statistical significance. Thus, no assertion about seasonality of infection can be made.

Seasonality is an important factor to examine, as presumably there is an insect or arachnid host of this *Trypanosoma* sp. transmitting it to these woylie populations (DEC, 2008). *T. lewisi* infection has been found to vary in the rat *Rattus norvegicus* with season as it affects flea population size (Linardi and Botelho, 2002). Interestingly, though, research has also shown that seasonal fluctuations of *Trypanosoma microti* exist in field voles (*Microtus agrestis*) even when direct transmission of the parasite, not flea-borne transmission, is the major mode of transmission between animals (Smith et al., 2006). Direct transmission, even when disease prevalence is low, can be an important epidemiological factor in the spread of disease, and this is an especially salient concern for a species whose conservation relies partially on relocations and reintroductions of possibly infected woylies to naïve populations.

Most likely, both insect-borne transmission and direct transmission are both contributing to the spread and prevalence of the trypanosome in woylie populations, and thus conservation efforts for the woylie need to address both vector-borne and direct transmission pathways of trypanosomes.
4.3 Limitations and Further Research

While addressing the possible significance of the data published here, it is necessary to acknowledge the limitations of the data. Sample sizes were small, ranging from 17 blood smears for the May 2009 Keninup population to 27 blood smears for the November 2009 Karakamia population. For seasonality assessments, slides from May 2009 as used for the Keninup population were not available from the Karakamia population; thus, data from blood smears was used from the closest lying month to May 2009 (September 2009). Microscopic diagnosis of infection with trypanosomes is not infallible; especially at low parasitemia levels as observed in the Keninup slides examined in this study, it would be easy to miss a trypanosome in 200 fields of view.

These limitations better inform ways to improve the study and possible avenues of future study. Ideally, microscopic morphological detection of trypanosomes would be paired with more accurate PCR methods of identifying trypanosome infection (as Smith et al. did in their 2009 paper). PCR can detect much lower levels of infection than microscopy, and thus can give a better idea of the prevalence of trypanosome infection within a population. Also, a study of *T. gondii* prevalence in the Karakamia and Keninup populations would help to determine whether a concomitant infection is responsible for the decline. A study on the ectoparasites found on woylies and the parasites they carry is underway, and these results could enlighten what insect or arachnid host can be a vector for the woylie’s *Trypanosoma* species.

5. Conclusion

Overall, microscopic inspection revealed that the prevalence and intensity of infection is higher in the woylie population from Keninup in the Upper Warren region, though individual intensity levels are low. From the data collected there also appears to be no statistically significant seasonal effect on trypanosome prevalence or intensity or infection. These results are similar to those discussed by Smith et al. in their 2009 paper investigating trypanosome prevalence and phylogeny in *B. penicillata*. While the data presented here cannot unequivocally indicate what the ultimate cause of the population declines seen in the Upper Warren region is, it does suggest that a higher prevalence of trypanosome infection,
most likely coupled with other biological and environmental features (e.g. *T. gondii* or stress), is potentially a factor in the decline.

This research can help formulate strategies to keep present woylie populations healthy. If feasible, it would be helpful to fence more woylie populations so they are not vulnerable to foxes, feral cats, and the diseases they might carry. Monitoring of both the stable population at Karakamia and the declining populations in the Upper Warren region should continue, and further research needs to be done on both trypanosome prevalence and other possible factors contributing to the declines. This would include further investigation of ectoparasites found on woylies to see if they are carriers of trypanosomes and research to see if *T. gondii* or condition-dependent expression of virulence causes greater adult mortality.

Hopefully, with more research on the cause of this decline, the woylie will once again serve as an example of how research combined with well-planned conservation can succeed.
Works Cited


