The epidemiology of Piroplasm Infection in the Woylie (Bettongia penicillata ogilbyi).

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Abstract

The woylie (*Bettongia penicillata ogilbyi*) is an endangered endemic species of the south-west of Western Australia that has experienced a 70-80% decline in the last five years. Among the potential agents for this event, infectious diseases are strongly suspected.

The aim of this study was to define the epidemiology of the haemoparasite, *Theileria penicillata*, in four localities: Karakamia, Keninup, Balban and Warrup. Light microscopy examination (LME) of 274 woylie blood smears was used in the study to establish *T. penicillata* parasitaemias (via count) and to detect any erythrocyte and leukocyte morphological changes. The protozoa prevalence and average parasitaemias (AP) were considered in relation to gender, location, and body condition; while AP was assessed in relation to haemoglobin (Hb) concentrations, haematocrit (HCT) and red blood cell (RBC) counts to evaluate possible clinical outcomes of the infectious agent.

The study highlighted previously unreported morphological findings of the erythrocytic cycle of *T. penicillata* in the woylie. Parasite infection did not account for any morphological alterations of the RBC and leukocytes. Piroplasm prevalence did not significantly vary between males and females, but was strongly associated with the locality of sampling. Higher and similar AP was detected in Balban and Keninup, while Warrup presented the lowest AP. AP was also strongly associated with Hb only rather than Hb, HCT and RBC count altogether. The reason why this occurred is uncertain and requires further investigation.

The establishment of fenced predator-free areas could assist in the study of the woylie biology as well as future conservation and management.

**Keywords:** piroplasm, woylie, decline, endangered species, wildlife disease, haematological profile, body condition
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1. Wildlife Extinctions and Their Underlining Causes.

Extinctions have been a constituent component of the evolutionary history on Earth (Pimm et al., 1995). Current rates though have worryingly escalated 100 to 1000 times in the last centuries, accelerating incredibly the process of biodiversity loss (Pimm et al., 1995; Smith et al., 2009). Establishing the causes of decline in a wildlife population is a fundamental step for efficacious conservation and management plans (Caughley, 1994). Nevertheless it is the most demanding task. A population decline can be triggered by multiple variables, whose interactions are possibly complex and not completely understood. Fragmentary knowledge of the demographic dynamic of the endangered species further complicates the challenge (Caughley, 1994).

The main driving forces implicated in the loss of biodiversity have been identified in the following categories: anthropocentric exploitation of the environment with habitat loss, fragmentation, pollution and over-harvesting of natural resources; introduction of non-native species; and loss of genetic variation (Pimm et al., 1995; Smith et al., 2009). The threat to wildlife by infectious agents may appear as a minor problem (Cleaveland et al., 2001). However, if combined to any of the previously mentioned factors, “there is substantial evidence that they can cause temporary or permanent decline in local species abundance” (Cleaveland et al., 2001).

Emerging infectious diseases have been at the centre of the public concern whenever wildlife has been implicated as reservoir of pathogens easily transmissible to humans and domestic animals (Daszak et al., 2000; Cleaveland et al., 2001). The opposite process though has gained consideration especially when managing endangered species (Daszak et al., 2000). The consequences of spill-over of diseases from domestic to wild populations and of introduction of new pathogens in naive populations cannot be underestimated anymore (Daszak et al., 2000).
2. **Disease Impact on a Wildlife Population.**

Several cases can be referred where infectious agents have concomitantly contributed to the decline and/or extinction of local wildlife populations (Smith *et al.*, 2006, 2009).

The Tasmanian tiger, *Thylacinus cynocephalus*, is an Australian example (Bulte *et al.*, 2003; Smith *et al.*, 2006, 2009). The thylacine was already rare or extinct on the Australian mainland before the arrival of the first settlers and survived only in Tasmania. The over-harvesting policy implemented by governmental bounties have been often blamed as major cause of its extinction, but a distemper-like disease could have acted in concert with the introduction of exotic species, such as the domestic dog, and human invasion of its habitat (Bulte *et al.*, 2003; Smith *et al.*, 2006, 2009).

The Tasmanian devil facial tumour (TDFT) is another illustration of how an infectious agent combined with habitat loss, over-exploitation and genetic impoverishment can drive a population to decline (Lachish *et al.*, 2010). The Tasmanian devil, *Sarcophilus harrisii*, has experienced a 60% decrease in its population size since 1996 and is presently at threat of extinction (McCallum, 2008). The risk of population extinction is inherent in its low numbers and is conceptually formalised by Caughley’s (1994) small population paradigm. The genetic diversity of an isolated population, as in the case of the Tasmanian devil, decreases with time, as a result of genetic drift (Caughley, 1994; Frankham, 2005). ‘Rare alleles that contribute little to heterozygosity are more easily lost during population size reductions’ (Lachish *et al.*, 2010). However also, reproduction between related animals (inbreeding) causes an increase in homozygosity and can be responsible for the fixation of deleterious traits over time (Caughley, 1994; Frankham, 2005). The loss of genetic diversity via genetic drift and inbreeding impacts negatively on the population fitness (inbreeding depression) and can ultimately influence the population’s ability to adapt to environmental changes (Caughley, 1994; Frankham, 2005). This self-perpetuating harmful cycle to potential extinction is summarised in Figure 1.
3. The Recent Decline of the Woylie.

The brush-tailed bettong (*Bettongia penicillata ogilbyi*) could become another Australian example of native species decline linked to infectious disease (DEC, 2008). Since 2001, the extant populations present in the southwest of Western Australia have experienced a rapid and intense decrease, estimated around 70-80% (DEC, 2008). Multi-factorial causes have been contributing to the woylie decline, such as habitat degradation and patchiness as well as introduction of feral cats and foxes (Maxwell *et al.*, 2008). Other elements that need to be considered are: alterations of fire regimes (Wayne *et al.*, 2008); variation in diet and food resource availability (Rodda *et al.*, 2008); rainfall and climatic fluctuations (Orell, 2008); and human intervention (‘trapping intensity, live harvest for translocation; trapping consequences: deaths, predations and pouch young intervention; and illegal killing/harvesting, and road kills’) (Wayne and Wilson, 2008).
The suspicion that infectious agents could be the cause of the recent decrease was raised based on the characteristic of the decline (Wayne in prep.). Figure 2 summarises the “untested hypothesis of the causes of woylie declines” by the Department of Environment and Conservation (DEC) (2008). Protozoal haemoparasites, like *Toxoplasma gondii* and *Trypanosome* spp., are under current investigation, as well as the potential impact of endoparasites and/or ectoparasites. Tick or flea burden could directly influence the health of the single animal or act as vectors for the transmission of other infectious agents (DEC, 2008). Additional “diseases considered being significant for future investigations include: Chlamydiales; Macropod Herpesvirus and Orbivirus; encephalomyocarditis virus; *Neospora caninum*” (Pacioni et al., 2008).

![Diagram](image)

Figure 2 The untested hypothesis of the causes of woylie declines in the Upper Warren region (WA) based on preliminary inferences (adapted from DEC, 2008). Legend: AOD = Agent of Decline; $\rightarrow$ = 1st order factor/higher confidence based on available evidence; $\rightarrow \rightarrow$ = 2nd order factor/likely relationship but less evidence available; stressors = predation; competition; climate factors/extreme weather; nutrition; high density woylie population; ectoparasites; disease reservoirs in sympatric species; concurrent infections.
This study focuses on the possible threat that a piroplasm infection, described recently by Clark and Spencer (2007), could directly or indirectly pose on the declining woylie population and it represents a component of the ongoing investigation on the potential role of infectious agents in the woylie decrease.


“Woylie” (stick carrier) is the Noongar word used to describe the ability of the brush-tailed bettongs (Betongia penicillata; Gray 1837) to carry leaves and sticks with their prehensile tail (Whitehurst, 1997). It is a small marsupial endemic to Australia and belonging to the Potoroidea family (Start et al., 1995; Groves, 2005). Its body length spans between 300 and 380mm, while its weight ranges between 1.1 and 1.6 Kg (Christensen, 2004).

Before the arrival of the first European settlers, the woylie inhabited most of the Australian

Figure 3 Historic distribution of woylies (Betongia penicillata) (adapted from Start et al., 1995).
continent south of the tropics, including the central desert areas, as determined from the Aboriginal oral history (Burbidge et al., 1988).

By the 1960’s though, the number of brush-tailed bettongs had rapidly declined, surviving only in the Perup/Lake Muir area, Tutanning Nature Reserve and Dryandra woodland, in WA (Wayne, 2008; Groom, 2010). Huge efforts have been directed towards woylie reintroductions in Western Australia, NSW and SA and fox control, in the 1970’s (Wayne, 2008). The creation, in 1996, of the ‘Western Shield’ conservation program has contributed dramatically to the recovery of the brush-tailed bettong, which then became the first Australian mammal to be downgraded in its protection status on Commonwealth and State conservation lists (Start et al., 1998). Since 2001, however, a new decline (up to 80%) has occurred in the remnant woylie populations, forcing the need to relist the species as ‘critically endangered’ in the IUCN Redlist (Wayne, 2008; Wayne et al., 2009). A concomitant decrease has also been recorded in the populations translocated in SA mainland, but the South Australian island populations appear to have remained relatively stable (Wayne, 2008).

The causes of the decline are yet to be determined and are at the heart of the Woylie Conservation and Research Project (WCRP), established by the Department of Environment and Conservation (http://www.dec.wa.gov.au/content/view/3230/97/ accessed 25/08/2010).

5. Brief Overview of the Woylie’s Ecological Functions.

“Research, especially in the last two decades, has led to a better understanding of the important ecological roles played by woylives” (Groom, 2010). The small marsupial is mycophagous. The main components of its diet are the fruiting bodies of hypogeous fungi, which it actively seeks by excavation. While digging, the brush-tailed bettong can displace on average 4.8 tonnes of soil annually, therefore improving incredibly soil and nutrient turnover, water penetration and ectomycorrhizal fungi dispersal (Lamont et al., 1985; Garkaklis et al., 1998, 2000, 2003). Whereas the seed-hoarding behaviour, the handling and storage of food items in hollows then covered by leaf litter or soil, aids seed dispersal, facilitates recruitment and regeneration of vegetation and ultimately influences fire regimes by constant reshaping of the understorey (Lamont et al., 1985; Murphy et al., 2005).
In the context of the ecological functions associated to the woylie, concerns may arise for “the far-reaching impacts on the ecosystem” that their decline could have (Groom, 2010).


Historically, multiple causes have contributed to the decline of the woylie. The habitat alteration that reshaped the Australian landscape since the arrival of the European settlers strongly restructured the environment available to the species (Burbidge and McKenzie, 1989; Start et al., 1995). Land clearing, livestock grazing and altered fire regimes have directly affected vegetation composition, leading to changes in the abundance, availability and/or suitability of resources such as water, food, shelter, reproductive mates and territory (Groom, 2010). The introduction of the European fox (*Vulpes vulpes*) and feral cat (*Felis catus*) has increased the rate of predation and hastened the marsupial decline, already hunted by native predators like Carpet Python (*Morelia spilota*) and large birds of prey (Burbidge and McKenzie, 1989; Start et al., 1995; Kinnear et al., 2002; Groom, 2010). Other biotic factors, such as food competition with the rabbit (*Oryctolagus cuniculus*) and other introduced grazing animals, must have escalated the process (Start et al., 1995). Abbott (2006) hypothesises that disease could have contributed to the decline of many Australian marsupials towards the end of the nineteenth century, but there is only anecdotal evidence, for this theory.

7. Piroplasm Taxonomic Classification.

The collective name ‘piroplasm’ describes the pear-shaped phenotype commonly observed in the erythrocyte of the mammalian host (Mehlhorn and Schein, 1984; Homer et al., 2000; Bowman, 2003; Lee, 2004; Uilenberg, 2006; Lee et al., 2009). Piroplasms are vector-borne intracellular protozoa (Allsopp et al., 1994). They belong to the Phylum *Apicomplexa*, Order *Piroplasmida*, which contains two main families, the *Babesiiidae* and *Theileriidae* (Homer et al., 2000; Morrison, 2009). The main morphological distinctive features of the phylum are the presence of an apical complex, specialised secretory organelles (rhoptries and micromene) and
8. Piroplasm Morphology.


The traditional classification of babesial and theilerial species has relied primarily on morphological, behavioural and biological characteristics (Lee, 2004). Phenotypic features, such as size, shape, and absence of pigment formation, have been easily identifiable from microscopic examination. Light microscopy of blood smears of infected animals has been the major diagnostic tool prior to the discovery of molecular biology techniques (Homer et al., 2000; Kunz, 2002; Lee, 2004).

Based on subjective investigation, babesial species have then been classified into ‘small’ and ‘large’ groups in relation to the pyriform body size (Homer et al., 2000; Lee, 2004; Morrison, 2009). The large Babesia spp. are characterised by intra-erythrocytic bodies whose length varies from 2.5 to 5 micron and is twice or five times the size of the so-called small Babesia spp., which instead measure between 1 and 2.5 micron. Babesia bigemina, B. major, B. caballi and B. canis are classic examples of ‘large’ Babesia; while B. bovis, B. ovis, B. gibsoni, B. felis and B. divergens are representatives of the ‘small’ Babesia (Barnett, 1977; Mahoney, 1977; Mehlhorn and Schein, 1984; Caccio’, 2002).

Theileria species have intraerythrocytic bodies of 1-2 micron (Mehlhorn and Schein, 1984; Homer et al., 2000; Lee, 2004). Theileria spp. cannot be reliably differentiated from ‘small’ Babesia spp. by LME (Irwin, 2009).

On morphological basis alone, the same protozoa could have presented with different morphological appearances in different hosts; or different phenotypes within the same host; or belonged to a different species but be phenotypically very similar in the same host (Homer et al., 2000; Kunz, 2002; Lee, 2004).
8.2. Shape.

As mentioned previously, the common name ‘piroplasm’ describes the prevalence of pear-shaped bodies in parasitised red blood cell (RBC). The haemoparasites are also rather pleomorphic. Their appearance in peripheral blood or bone marrow varies, as represented in Figure 4 (Backhouse and Bollinger, 1957; Clark et al., 2004).

The *Theileria* spp. may be found as comma, bayonet, round, ovoid, rod-like, amoeboid or irregularly shaped in erythrocytes and lymphocytes (Barnett, 1977; Clark et al., 2004). *Babesia* spp. are equal or relatively larger and round, pyriform or irregular and only within the RBC (Barnett, 1977; Clark et al., 2004). This variety of shapes could help distinguish the two genera, but individual piroplasms are impossible to differentiate (Barnett, 1977). The elongated bacillary or bayonet forms are typical of *Theileria* as are the tetrad or Maltese cross (Barnett, 1977).
Figure 4 Line drawings of piroplasms of echidna, *Babesia tachyglossi* and *Theileria tachyglossi*, which show the range of parasites forms that may be encountered. **Figures 1-30.** Forms of the parasites seen in RBCs and bone marrow of echidna. **Figures 1-5.** Commonly occurring forms. Particularly frequent are marginal forms as in Fig. 4. Often thicker than that shown and reminiscent of the ‘appliquée’ form of malarial plasmodia. **Figure 6-10.** Larger pleomorphic forms as observed mainly in echidna 2. **Figures 11-15.** Blood of echidna 3. Small, slender, wispy, ‘duplex’ forms as in Fig. 13 and Fig. 15 were present. **Figures 16-20.** Large forms seen only in bone marrow smears of echidna 3. Bigeminate Babesia-like organisms as in Fig. 16 were common. Cells containing from four to eight parasites (Figs. 17 and 18) and curved attenuated structures (Figs. 19 and 20) were also features of this animal. **Figures 21-24.** Minute forms in blood of echidna 6, which were numerous. In the bone marrow occasional red cells contain larger ovoid forms as in Fig. 25. **Figures 26-30.** Echidna 10. A heavy infection exhibiting many minute forms as well as those depicted (adapted from Backhouse and Bollinger, 1957, and Clark et al., 2004).
8.3. Absence of Pigment Formation.

The other common feature of *Babesia* and *Theileria* is the absence of pigment production (Mehlhorn and Schein, 1984; Uilenberg, 2006). The two genera digest completely the haemoglobin of the targeted erythrocyte, without leaving any residue, although some *Theileria* species (*T. velifera, T. separata, T. buffeli*) partially digest haemoglobin, forming crystallised compounds. This feature distinguishes the Piroplasmida order from genera like *Plasmodium* and *Haemoproteus* (Uilenberg, 2006).

8.4. Piroplasm Life Cycle: Biological Similarities and Differences.

The life cycle of Apicomplexans is quite complex. It consists of three reproductive stages (gamogony, sporogony and merogony), and relies on intermediate and definitive hosts (Barnett, 1977; Mahoney, 1977; Homer *et al.*, 2000). Gamogony is a form of sexual reproduction. It occurs in the gut of the intermediate vector, normally a tick of the genus *Ixodid*, and aims to the formation of gametes, which then fuse inside the non-vertebrate host. Sporogony is an asexual duplication, which occurs in the vector salivary glands. Merogony is another asexual replication that takes place in the vertebrate host (Mehlhorn and Schein, 1984; Homer *et al.*, 2000).

The classical biological differences between *Theileria* and *Babesia* life cycles are: the absence in *Babesia* species of a pre-erythrocytic schizogonic replication in the mammalian host, which is instead distinctive of the theilerial genus; and the spread of *Babesia* from one infected tick generation to the following one via transovarial transmission. This vertical transmission is absent in *Theileria*. The protozoan sporogonic multiplication occurs exclusively at the vector’s salivary gland (Barnett, 1977; Mahoney, 1977; Mehlhorn and Schein, 1984; Homer *et al.*, 2000; Uilenberg, 2006).

In *Babesia* the division into the definitive host’s RBCs occurs by budding and often results in two pyriform daughter cells (merozoites), while in *Theileria* up to four or more merozoites are formed within the parasitized erythrocyte, giving rise to the typical Maltese cross disposition (Uilenberg, 2006).
The life cycle of *Theileria* species is described in more detail in the following paragraph, while a genus general example is visually represented in Figure 5.

### 8.5. *Theileria* species Life Cycle.

Sporozoites are transmitted to the definitive host’s blood stream by inoculation of infected saliva from a feeding tick (Barnett, 1977; Mehlhorn and Schein, 1984; Homer *et al.*, 2000; Uilenberg, 2006; Lee, 2004). The non-motile sporozoites come into contact with a circulating...
leukocyte, macrophage or lymphocyte, by pure chance. Once attached, they penetrate the host cell by a gradual circumferential ‘zippering’ and develop into multinucleated syncytial schizonts (Shaw, 2003). A percentage of schizonts divide (merogony) and form merozoites that are then released in the blood stream and invade additional RBCs (Barnett, 1977; Mehlhorn and Schein, 1984; Homer et al., 2000; Uilenberg, 2006; Lee, 2004). Within the host erythrocytes, the merozoites described in previous sections, undergo a further binary fission, forming the typical non-pigmented pear-shaped bodies (Homer et al., 2000; Uilenberg, 2006).

A new tick becomes infected after ingesting a blood meal containing RBC with merozoites. The infected erythrocytes are digested and lysed in the tick gut, releasing the piroplasms (Mehlhorn and Schein, 1984; Homer et al., 2000; Uilenberg, 2006). At this stage the protozoa can undertake gametogony, producing macrogametes and microgametes, which then fuse forming a motile zygote (ookinete). Ookinetes migrate and reach the tick’s salivary gland to undergo asexual reproduction, without invading any other organs (Mehlhorn and Schein, 1984; Homer et al., 2000). Tick feeding initiates rapid sporogony in the salivary glands, and infective sporozoites are injected during the later stages of the blood meal (transstadial transmission), restarting the life cycle (Shaw, 2003).

The tick loses its theilerial infection after having transmitted it. The infection does not endure to the next stage or even to the next generation (Mehlhorn and Schein, 1984; Uilenberg, 2006). “When the larva becomes infected the nymph is infective, when the nymph is infected the adult tick is infective. Newly hatched larvae are never infected or infective” (Uilenberg, 2006).


As mentioned previously, the babesial sporozoites are transmitted to a new vertebrate host by infected tick saliva, as observed with Theileria species. Almost immediately they invade the host’s RBC to produce the pear-shaped merozoites, without any intra-lymphocytic replication. Once a new vector attaches to the infected definitive host and ingests the circulating merozoites, the gametogonic reproduction occurs, exactly as seen in Theileria species. In
Babesia, though, the ookinetes enter various organs, including salivary glands and ovaries, to undertake sporogony. In this way the infection is passed by vertical transmission to the next tick generation. When female ticks become infected, sporogony takes place in the salivary glands of larval, nymphal and- or adult ticks of the following generation (Mehlhorn and Schein, 1984; Homer et al., 2000; Uilenberg, 2006). Certain species of Babesia can carry on over numerous tick generations, even without new infections (Uilenberg, 2006).


Comprehensive reviews on protozoan parasites in Australian marsupials are sparse and this becomes particularly evident when compared to studies on piroplasms infecting domesticated animals such as companion animals and livestock (O’Donoghue, 1997; Lee, 2004). The observation of Babesia and Theileria species in native Australian wildlife has been mainly associated to incidental findings or individual case reports (O’Donoghue, 1997; Lee, 2004). Very little is known about the actual incidence of naturally occurring protozoan infections and their impacts on the native fauna (O’Donoghue, 1997).

The first identification of a piroplasm in an Australian animal dates back to 1915 (Priestly, 1915). Priestly (1915) consistently found an intra-erythrocytic parasite in blood films from the short-beaked echidna (Tachyglossus aculeatus). He would later name it Theileria tachyglossi (Priestly, 1915). Since then, knowledge of the range of infected marsupials has widened, as has the identification of new piroplasms. From the first isolations in the monotremes, the short-beaked echidna and the platypus (Ornithorhynchus anatinus), up to ten marsupials have been reported to present Babesia and/or Theileria-like organisms in blood or tissue samples (Priestly, 1915; Backhouse and Bollinger, 1957; Mackerras, 1958; Mackerras, 1959). The most recent investigations have been conducted by Clark and Spencer (2007) and Lee (2004, 2009). Clark and Spencer (2007) identified and named three new Theileria species in three distinct macropods. They are: Theileria brachyuri in the quokka (Setonix brachyurus); Theileria penicillata in the brush-tailed bettong (Bettongia penicillata ogilbyi); and Theileria fuliginosa in the Western grey kangaroo (Macropus fuliginosus). Theileria
gilberti n.sp. is the name recommended for the protozoa described from the Gilbert’s potoroo (*Potorous gilbertii*) (Lee, 2004; Lee *et al.*, 2009). Table 1 lists the piroplasm infections reported in Australian native animals to date (2010).

Table 1 Overview of the reported cases of piroplasm infection in Australian native animals (adapted from: O’Donoghue and Adlard, 2000; Clark *et al.*, 2004; Lee, 2004).

<table>
<thead>
<tr>
<th>Host species</th>
<th>Piroplasm species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platypus (<em>Ornithorhynchus anatinus</em>)</td>
<td>Theileria sp. Theileria ornithorhynchi</td>
<td>Mackerras, 1958; Mackerras, 1959; McMillan and Bancroft, 1974</td>
</tr>
<tr>
<td>Short-beaked echidna (<em>Tachyglossus aculeatus</em>)</td>
<td>Babesia sp. Theileria tachyglossi</td>
<td>Backhouse and Bollinger, 1957; Mackerras, 1959; Priestly, 1915; Seddon, 1952; Mackerras, 1959; Seddon and Albiston, 1966</td>
</tr>
<tr>
<td></td>
<td>Babesia tachyglossi</td>
<td>Backhouse and Bollinger, 1957; Ristic and Lewis, 1977</td>
</tr>
<tr>
<td>Brown antechinus (<em>Antechinus stuartii</em>)</td>
<td>Babesia sp.</td>
<td>Arundel <em>et al.</em>, 1977</td>
</tr>
<tr>
<td>Northern brown bandicoot (<em>Isoodon macrourus</em>)</td>
<td>Theileria sp.</td>
<td>Seddon and Albiston, 1966</td>
</tr>
<tr>
<td>Southern brown bandicoot (<em>Isoodon obesulus</em>)</td>
<td>Babesia thylacis Theileria peramelis</td>
<td>Mackerras, 1959; Mackerras, 1959; Munday, 1978, 1988; Mackerras, 1959</td>
</tr>
<tr>
<td>Long-nosed bandicoot (<em>Perameles nasuta</em>)</td>
<td>Theileria sp. Theileria peramelis</td>
<td>Mackerras, 1958; Munday, 1978, 1988; Mackerras, 1959</td>
</tr>
<tr>
<td>Long-nosed potoroo (<em>Potorous tridactylus</em>)</td>
<td>Theileria sp. Theileria peramelis</td>
<td>Mackerras <em>et al.</em>, 1953; Mackerras, 1958; Munday, 1978; Speare <em>et al.</em>, 1989</td>
</tr>
<tr>
<td></td>
<td>Theileria peramelis</td>
<td>Mackerras, 1959</td>
</tr>
<tr>
<td>Proserpine rock-wallaby (<em>Petrogale Persephone</em>)</td>
<td>Babesia sp.</td>
<td>O’Donoghue, 1997</td>
</tr>
<tr>
<td>Quokka (<em>Setonix brachyurus</em>)</td>
<td>Theileria brachyuri</td>
<td>Clark and Spencer, 2007</td>
</tr>
<tr>
<td>Brush-tailed bettong (<em>Bettongia penicillata ogilbyi</em>)</td>
<td>Theileria penicillata</td>
<td>Clark and Spencer, 2007</td>
</tr>
<tr>
<td>Western grey kangaroo (<em>Macropus fuliginosus</em>)</td>
<td>Theileria fuliginosa</td>
<td>Clark and Spencer, 2007</td>
</tr>
<tr>
<td>Gilbert’s potoroo (<em>Potorous gilbertii</em>)</td>
<td>Theileria gilberti n.sp.</td>
<td>Lee <em>et al.</em>, 2009</td>
</tr>
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10. Piroplasmosis Diagnosis.

10.1. Microscopy.

Microscopic examination of stained blood smears is the routine protocol for the diagnosis of piroplasmosis (Barnett, 1977; Mahoney, 1977; Homer et al., 2000; Clark et al., 2004; Lee, 2004). Depending upon the magnitude of the parasitaemia, the merozoites should be detectable within the host’s red blood cells (Clark et al., 2004; Lee, 2004). Their morphology varies from the classic ‘ring with a stone’ appearance or the tetrads (Maltese cross) in *Theileria*, to single or paired pear-shaped merozoites in the *Babesia* genus (Homer et al., 2000). As described previously, the assumption that absence of lymphocytic schizogony implies babesial piroplasmosis only is rather misleading (Uilenberg, 2006). The extrerythrocytic multiplication that *Theileria* species undertake before invading the RBCs is difficult to find, even in some unquestionable species of *Theileria* (Uilenberg, 2006). Phagocytosis of an infected RBC by a macrophage could be misinterpreted as intralymphocytic schizogony (Callow, 1984).

The analysis of blood films is a subjective process, which relies on the experience and knowledge of the observer and a reasonable amount of time spent to examining the smear (Homer et al., 2000). Regardless, as noted earlier, an absolute discrimination between genera and species is unreliable if exclusively based on morphological resemblance, even for the most experienced parasitologist (Barnett, 1977; Mahoney, 1977). Low parasitaemias (i.e., less than 0.1% of erythrocytes infected), are difficult to detect by routine microscopy, hence the need to combine light microscopy inspection with complementary tests, such as serology or, as illustrated in more detail in the following paragraph, molecular technologies (Callow, 1984; Gaunt, 2000; Homer et al., 2000; Lee, 2004).

10.2. Polymerase Chain Reaction (PCR) Diagnosis.

In the last two decades, molecular techniques, such as polymerase chain reaction (PCR), have been more often used to complement microscopic screening of blood films in the detection and identification of haemoproteozoa (Kunz, 2002; Lee, 2004).
PCR is an extremely sensitive method capable to assess parasitaemias of *Babesia* sp. of approximately 0.000003% (Jefferies *et al.*, 2003). The fault associated to such high sensitivity is the likelihood of false positives and negatives (Yang and Rothman, 2004). The “background contamination from exogenous sources of DNA is a common cause of false-positives [...] the “carryover” of products from earlier PCR reactions can be harboured and transmitted through previous PCR reagents, tubes, pipettes, and laboratory surfaces.” (Yang and Rothman, 2004). “Very minor amounts of carry-over contamination may serve as substrates for amplification and lead to false-positive results.” (Yang and Rothman, 2004). False-negative findings are instead associated with the concentration and purification of the DNA sample prior to the beginning the amplification process (Yang and Rothman, 2004). “Three of the most commonly encountered problems are in fact: inadequate removal of PCR inhibitors in the sample, such as haemoglobin, blood culture media, urine, and sputum; ineffective release of microbial DNA content from the cells; or poor DNA recovery after extraction and purification steps.” (Yang and Rothman, 2004).

The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions (Yang and Rothman, 2004). A schematic representation of the three major steps in each PCR cycle is illustrated in the following figure. As PCR progresses, the DNA template is exponentially amplified (Yang and Rothman, 2004).
The PCR reaction takes place in a thermocycler. Each PCR cycle consists of three major steps: (1) denaturation of template DNA into single-stranded DNA; (2) primers annealing to their complementary target sequences; and (3) extension of primers via DNA polymerisation to generate new copy of the target DNA. At the end of each cycle the newly synthesised DNA act as new targets for the next cycle. Subsequently, by repeating the cycle multiple times, logarithmic amplification of the target DNA occurs (adapted from Yang and Rothman, 2004).

10.3. Molecular Tools for Phylogenetic Analysis.

The use of PCR to amplify and sequence highly conserved genes is a more objective technique in species characterisation than morphology and life history (Barta, 2001; Kunz, 2002; Criado-Fornelio et al., 2003). In the case of protists, some of the highly conserved genes are the sequences associated to the subunit 18 of the ribosomal RNA (18SrRNA) genes (Barta, 2001; Caccio’, 2002). These are most commonly used to infer evolutionary correlation on a molecular basis (Barta, 2001; Caccio’, 2002; Schnittger et al., 2003).

The application of molecular technology to redefine traditional taxonomy has not been without frictions or confusion (Kunz, 2002) and the Order Piroplasmida has not been left out of this debate. In recent years, a new family, the Nicollidae, has been proposed to include Babesia equi, B. rodhaini and Cytauxzoon felis, which have intermediate properties of the two genera (Allsopp et al., 1994). Other studies have suggested up to three or four new taxa of
piroplasms based on the sequencing of 18SrRNA from various babesial and theilerial isolates (Criado-Fornelio et al., 2003).

It has been suggested that the piroplasms are quite ancestral haemoparasites (Criado-Fornelio et al., 2003). They must have developed in their mammalian or arthropod host in Africa, during the Pleocene, around 60-55 million years ago (Criado-Fornelio et al., 2003).

The current biogeographical knowledge of piroplasms is still incomplete, lacking the sequencing of species from South America and Australia. More comprehensive approaches and techniques will improve the final evolutionary hypothesis (Criado-Fornelio et al., 2003).


Clark and Spencer (2007) have described *Theileria penicillata* as a new species “during an assessment of blood samples as part of an investigation of animal health” in the woylie. A predictable finding has been the impossibility to distinguish *T. penicillata* from other piroplasms merely on a morphological basis (Clark and Spencer, 2007). The pleomorphic aspect and the reduced size, 0.4-1.2 µm x 0.8-1.5 µm, were not indicative of the species when compared to the piroplasms in other Macropodoidea (Clark and Spencer, 2007). By relying on molecular analysis though, these authors have ascertained the monophyletic origin of the organism within the *Theileriidae* family and suggested the new species name (Clark and Spencer, 2007).
12. Objectives and Aims of the Study.

The specific aims of this study were:

a. To describe morphological variations of (i) the piroplasms and (ii) the RBC and WBC by LME in selected blood films.

b. To investigate piroplasm prevalence by gender and location.

c. To estimate AP and determine its correlation with woylie gender, location and body condition.

d. To estimate AP variation for Keninup, pre- and post-decline.

e. To relate AP to Hb, HCT and RBC count.

f. To correlate haemolysis of the whole blood samples to piroplasm infection or alternative causes.
13. **Material and Methods.**

13.1. **Blood Samples.**

A total of 274 blood smears (112 female and 162 male adult woylies) were selected for this study from the woylies trapped in Karakamia Sanctuary and in the Upper Warren region between 2006 and 2009 as part of WCRP (Pacioni, 2010). Only adults were included in the study, excluding any juveniles or sub-adults.

Blood samples were collected from the lateral caudal vein and preserved in EDTA in commercial tubes (as described by Pacioni, 2010). Between 2 to 3 blood films per animal were prepared and air dried in the field, while the whole blood samples were submitted to Murdoch University Clinical Pathology Laboratory (MUCPL) within 36 hours from collection and processed (Pacioni, 2010). Morphology of cells, including examination for red blood cell parasites, was assessed (Pacioni, 2010). Blood smears were stained with Wright’s/Giemsa stain soon after collection (Pacioni, 2010) however a few unstained ones were submitted to Murdoch University Clinical Pathology Laboratory to be processed with Wright’s/Giemsa stain in September 2010 for this study. The number of blood smears screened classified by gender and location is summarised in Table 2.

<table>
<thead>
<tr>
<th>LOCATION</th>
<th>FEMALE</th>
<th>MALE</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balban</td>
<td>7</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>Karakamia</td>
<td>13</td>
<td>13</td>
<td>26</td>
</tr>
<tr>
<td>Keninup</td>
<td>59</td>
<td>77</td>
<td>136</td>
</tr>
<tr>
<td>Warrup</td>
<td>33</td>
<td>50</td>
<td>83</td>
</tr>
<tr>
<td>GRAND</td>
<td>112</td>
<td>162</td>
<td>274</td>
</tr>
<tr>
<td>TOTAL</td>
<td>112</td>
<td>162</td>
<td>274</td>
</tr>
</tbody>
</table>

Our study referred to the haematological profiles processed by MUCPL between 2006 and 2009 (as described by Pacioni 2010) when analysing Hb, HCT and RBC count. The samples that were haemolysed before reaching the laboratory were identified and a correlation to piroplasm infection or alternative causes was considered in our study.
13.2. Woylies’ Population Locations.

Four locations were chosen: Balban, Karakamia, Keninup and Warrup. Their selection was based on: the animals’ density per single location (close to or below carrying capacity), which warranted a representative sample size; and the variation of these same densities by location over time and at different stages of the decline.

The specific locations are visualised in the following figures (Figure 7 and 8).

Karakamia Sanctuary is located 50 Km North-East of Perth. The woylie population here resident extends on a 275 ha property that is completely predator-proof (Pacioni, 2010). Originally the land was used for livestock grazing and Jarrah timber production, but the majority of the sanctuary has kept or restored its original Jarrah forest (Eucalyptus marginata) and mixed woodlands of Marri (Corymbia calophylla) and Wandoo (E. wandoo) (Pacioni, 2010).

Keninup and Balban are situated in the eastern side of the Upper Warren region, 300K Km in the south-west of Western Australia, while Warrup belongs to the western side of the area. The vegetation is characterised by Karri forest (E. diversicolor), interspersed by Jarrah and Marri woodland (Pacioni, 2010).
Figure 7 Karakamia and the Upper Warren region: locations of the woylie populations selected by this study (adapted from Wayne, 2008).

Figure 8 Keninup, Balban and Warrup locations in the Upper Warren region (adapted from Pacioni, 2010).
13.3. Light Microscopy Examination of the Blood Films.

Every blood smear was examined under light microscopy in accordance to the following protocol:

1. The sample identification number was noted and recorded

2. First, 5-10 fields at 200x magnification were examined in the blood film, to estimate any qualitative or quantitative abnormality of RBC and/or white blood cells (WBC), such as signs of regenerative anaemia or increase/decrease of WBC

3. At 400x magnification 5-10 fields were examined to establish presence/absence of piroplasms within the RBC, noting down any other haemoparasites encountered, such as *Trypanosoma* spp., or other interesting morphological findings

4. With the oil-immersion (100x) objective lens, 50 high power (HP – x1,000) fields for the piroplasm-negative slides and 100 HP fields for the piroplasm-positive samples were examined, and each piroplasm was counted (see below)

5. Digital photographs were taken of the protozoa and host cells to create an image library.

13.4. Parasite Counts (Estimation of Parasitaemia).

“An estimate of the degree of infection is established by enumerating the parasites in blood smear in relation to RBC” (Callow, 1984). At HP magnification all the piroplasms present in 100 fields were counted. All the RBC present in 10 fields were then counted, always at HP. The percentage of RBC infected by piroplasm equalled the total number of parasites (from 100 fields) divided by the total number of RBC (average number of RBC counted in 10 fields multiplied by 10) (Callow, 1984).
13.5. Data Analysis.

The statistical analysis was performed using SPSS 17.0 (SPSS Inc., 2008).

To evaluate potential relationships among the three categorical variables, piroplasm prevalence, woylie gender and population locations, a loglinear analysis has been conducted.

The normality of the AP was inspected and the Kolmogorov-Smirnov test and both z-skewness and z-kurtosis were conducted to confirm a non normal distribution. The AP was transformed via square root transformation to obtain a normal distribution. The fit of regression model for the transformed parasitaemiae, gender and location variables were then assessed using multiple regression and ANOVA. A one-way ANOVA analysis was used to detect the differences in levels of AP in relation to the different locations, while a Spearman’s test was performed to detect potential influence of parasitaemia on the animals’ body and coat conditions in each single location.

An independent t-test has been performed to evaluate a longitudinal study of piroplasm parasitaemia in the remnant woylie population present in Keninup.

A loglinear analysis was performed to correlate haemolytic blood samples to the presence or absence of the haemoparasite and in relation to the different locations.

Finally, multiple linear regressions were performed to establish correlations between the square root of the AP and haemoglobin concentration, haematocrit levels and red blood cell counts.
14. Results.


While estimating average parasitaemia (AP), a variety of morphological features were noted, such as pleomorphism of the *Theileria* sp., as well as unreported findings regarding *Theileria penicillata* morphology. Some digital photographs of *T. penicillata* intra-erythrocytic infection in the brush-tailed bettong are reproduced below (Figures 9 to 13). Other findings are included, such as detection of *Trypanosoma* haemoparasites, shown in Figure 14 and 15.

No significant RBC and WBC morphological abnormalities were encountered in the light microscopy examination (for example, there were no signs of regenerative anaemia or leukocytosis).
Figure 9 *Theileria penicillata* (arrow) dividing within woylie’s RBC: Maltese cross feature. Bar = 10 µm (photo by Stefania Basile).

Figure 10 *T. penicillata* (arrow) with the typical ‘stone and a ring’ shape in proximity of a Howell Jolly body. Bar = 10 µm (photo by Stefania Basile).

Figure 11 *T. penicillata* (arrow) with the ovoidal shape. Bar = 10 µm (photo by Stefania Basile).

Figure 12 *T. penicillata* (arrow) with the ‘bayonet’ shape. Bar = 10 µm (photo by Stefania Basile).

Figure 13 Dividing forms of *T. penicillata* (arrow) within a RBC. Bar = 10 µm (photo by Stefania Basile).
Figure 14 *Trypanosoma* sp. encountered at LME (Bar = 10µ) (photo by Stefania Basile).

Figure 15 *Trypanosoma* sp. with its undulating membrane across the middle (photo by Stefania Basile).
14.2. Piroplasm Prevalence by Gender and Location.

The piroplasm prevalence by gender and location is summarised in Table 3. Higher prevalence was recorded for Keninup, while Balban, Karakamia and Warrup had similar overall lower prevalences. The two-way loglinear analysis generated a final model that retained only two effects. The K-way Effects likelihood ratio of the model had Chi-square = 102.405, df = 7 and p < 0.0005. This indicated that the variables piroplasm prevalence x location was highly significant, when compared to the prevalence x gender interaction, which was not significant (p > 0.05).

Table 3 Piroplasm prevalence (%) by gender and location

<table>
<thead>
<tr>
<th>LOCATION</th>
<th>FEMALE PREVALENCE (%)</th>
<th>MALE PREVALENCE (%)</th>
<th>TOTAL PREVALENCE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balban</td>
<td>2.5</td>
<td>8.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Karakamia</td>
<td>6.4</td>
<td>6.4</td>
<td>12.8</td>
</tr>
<tr>
<td>Keninup</td>
<td>20.6</td>
<td>27</td>
<td>47.6</td>
</tr>
<tr>
<td>Warrup</td>
<td>2.9</td>
<td>5.4</td>
<td>8.3</td>
</tr>
</tbody>
</table>

14.3. Piroplasm Parasitaemia Correlation to Gender, Location and Body Condition.

The multiple regression model revealed that population location accounted for 32% of the variability of the transformed AP, while gender had no influence at all. The ANOVA indicated that AP is strongly correlated to location (p < 0.05) rather than gender. In the one-way ANOVA, the homogeneity of variances of transformed AP by location has been met (Leven’s test was not significant, p > 0.05), while the transformed AP among locations were significantly different (ANOVA test, p < 0.0005). Hochberg’s post-hoc test highlighted a significant difference (p < 0.0005) among the transformed AP of the various locations, with the highest and similar levels in Balban and Keninup, followed by Karakamia, while the
transformed AP of the woylies at Warrup were significantly lower. This is summarized in Figure 16.

![Figure 16 AP by location.](image)

The Spearman’s test was not significant (p < 0.05) for every location, underlining that the transformed AP were not affecting woylies’ body condition and coat status.

14.4. Piroplasm Parasitaemia Variation in Keninup over Time.

There was no significant (p > 0.05) difference in transformed AP in woylie cohorts before and after the decline at Keninup, yet there was a tendency for the transformed AP to be higher before the decline (M= 6.5; SE= 0.45) compared with later (M= 5.9; SE= 0.27).
14.5. Piroplasm Parasitaemia Correlation with Haemoglobin (Hb), Haematocrit (HCT) and RBC Counts.

The two-way loglinear analysis generated a final model that retained only two effects. The K-way Effects likelihood ratio of the model had Chi-square = 117, df = 10 and p < 0.0005. This indicated that the two-way interaction (haemolysis x location) was highly significant when compared to the haemolysis x piroplasm infection interaction, which was not significant (p > 0.05). These results suggest that the parasite infection was not responsible of the haemolysis of some of the blood samples collected, but rather the blood collection procedures in some locations could have not been optimal.

The multiple regression assessed that Hb accounted for 15.2% of the variability of the transformed AP, while HCT and RBC accounted only for 4.3% and 6.8% respectively. The ANOVA indicated that the transformed AP is strongly correlated to Hb (p < 0.005) rather than HCT and RBC (p > 0.05).
15. Discussion.

15.1. Light Microscopy.

The LME findings on *Theileria penicillata* in the woylie confirmed the previously reported information about the morphology of this parasite (Clark and Spencer, 2007). *T. penicillata* is a small pleomorphic piroplasm (0.4-1.2 µm x 0.8-1.5 µm) (Clark and Spencer, 2007). The variation in shape described by the same authors (Clark and Spencer, 2007) was also observed in this study (Figure 11, 12 and 13). An interesting discovery was the detection of the characteristic ‘Maltese cross’ during the erythrocytic cycle of the piroplasms in the RBC. Observation of this morphological form could be predicted on the basis of what is known about the normal reproductive behaviour of the *Theileriidae* family, but was confirmed during this project by frequent observation of the form during LME (Figure 9). Another finding was the presence of multiple ‘ring-with-a-stone’ protozoa within the same erythrocyte. These presented with differing sizes (Figure 13) and it is hypothesised that these are simply morphological variations of *T. penicillata* which naturally occur within the species and most likely reflects normal maturation of the parasite and different trophozoite stages. Alternatively, the different morphological forms could represent different *Theileria* spp., or instead be *Babesia* spp. The impossibility to distinguish *T. penicillata* from other piroplasms merely on a morphological basis has been previously discussed (Clark and Spencer, 2007; Irwin, 2009) and molecular analysis is necessary to ascertain the monophyletic origin of the parasite(s) (Clark and Spencer, 2007).

Occasional findings of *Trypanosoma* parasites was also reported (Figure 14 and 15), suggesting multiple haemoparasite infections in individual woylies. Further investigation is needed to confirm the identity of these parasites, but the morphology was consistent with previously reported trypanosomes observed in the blood of woylies in the Upper Warren region (where these samples were obtained during allied studies under the WRCP) (Smith *et al.*, 2009).
15.2. Statistical Analysis.

15.2.1. Piroplasm Prevalence by Gender.

In our study, there was no significant difference in piroplasm prevalence between male and female woylies, contradicting the findings of Wilson et al. (2002) in other species. These authors described the tendency of male mammals to be more prone to higher parasite burdens due to their behaviour; males are more likely than females to cover larger home ranges for territorial, reproductive and feeding purposes (Wilson et al., 2002). This behaviour was confirmed in the case of male woylies by Pacioni (2010); where females had the tendency to remain within or close to their mother’s home range (less than 1km), males were more mobile, dispersing up to 3km (Pacioni, 2010). Longer distances (more than 6km) were also covered by male woylies but more rarely (Pacioni, 2010). In spite of behavioural differences between the two woylie genders, variation in home ranges did not seem to affect the prevalence of piroplasms in males and females in our study, highlighting that other factors may contribute to this such as the distribution and/or density of the (presumed) intermediate host, the Ixodid tick.

Establishing piroplasm prevalence across age groups, including sub-adults and juveniles for examples, should be investigated to broaden our knowledge of the parasite epidemiology. The challenge, though, will be to tailor capture techniques for these categories. Females with pouch young should be also included in the demographic study but the ethical issue of pouch young rejection by the mother and consequent pouch young survival should not be underestimated.

15.2.2. Is Piroplasm conducive to the Woylie Decline?

As Pacioni described (2010) there is a spatial pattern in the decline of the woylie. The eastern regions of the Upper Warren (Balban and Keninup) were the first to report a decrease; then the decline progressed north at a rate of 5-10 Km per year (Pacioni, 2010). This spatial model is supported by the fact that Karakamia is the only location in WA to be still stable over time.
The pattern of decline may mimic the spreading of an infectious agent, from the southern to the northern regions (Pacioni et al., 2010). However, our findings do not support this hypothesis. If piroplasm infection was the causative agent of the decline, still to be experienced in the northern location (Karakamia), AP in Karakamia might be predicted to be significantly lower than those in Balban and Keninup. This was not the case; northern (Karakamia) and southern (Balban and Keninup) locations presented with almost equal AP. Furthermore, no cytological evidence of red cell insult (for example, regenerative changes within erythrocytes suggesting anaemia) was detected in any of the samples examined. Although it might be argued that only healthy individuals were sampled, it seems unlikely that at least some indication of red cell insult was not found if the parasites were causing illness. Possible conclusions that we could derive from this are: (i) piroplasm infection is not the primary decline drive; (ii) piroplasm could interact synergistically with other elements (climate, nutrition, predation, fire) and be conducive to decline in the long term.

The Theileriidae family is an ancient group of parasites (Criado-Fornelio et al., 2003). It must be presumed that the host-parasite relationship between T. penicillata and Bettongia penicillata olgilbyi has co-evolved over many millennia, potentially establishing equilibrium. This could be supported by the AP in woylies trapped in Keninup pre- and post-decline, which did not vary significantly. Investigating similar parameters in Balban, Warrup and Karakamia would be interesting, but inadequate sample sizes were available. Similarly estimating prevalence and AP of piroplasm in the woylies translocated in South Australia could represent an important term of comparison.

As mentioned previously, concomitant intrinsic or extrinsic causes are more likely to perturbate the host-parasite balance and affect the woylie population fitness (Spalding and Forrester, 1993; Wobeser, 2007).

Subtle effects of an infectious agent on survival and/or fecundity could have important consequences on the woylie population (Spalding and Forrester, 1993; Wobeser, 2007). For example, the cow-pox virus in rodents is asymptomatic and does not influence the new born survivorship. The infection, however, delays the reproduction onset by one month, reducing by 25% the reproductive output in such short-lived animals (Wobeser, 2007). A mild dysfunction due to sub-clinical or sub-lethal disease could indirectly influence survival by
increasing the vulnerability to predation or other factors (Spalding and Forrester, 1993; Wobeser, 2007).

In regards to predation, Karakamia represents a peculiar setting when compared to the other locations. It is a vermin-proof area, where the woylies reached their carrying capacity (400-600 subjects), and remained stable during the decline (Pacioni, 2010). Analogously, this time due to geographic isolation, there is also an absence of introduced predators in the South Australian islands (Pacioni, 2010). The translocated island population, similarly to the protected Karakamia woylies, remained constant over time (Pacioni, 2010). This has some implications for future management. As Finlayson et al. (2008) suggested secure and self-sustaining populations of threatened species, like the woylie, through the establishment of fenced areas could assist in the study of the woylie biology as well as future conservation and management.

When considering the general wellbeing of the animals, body and coat condition were not affected during and after the decline (Pacioni, 2010) and were not significantly associated to AP. The next reliable index of wildlife general health status was haematology (Clark, 2004). In our study Hb, HCT and RBC count were considered.

Critical to this discussion about potential pathogenesis of *Theileria penicillata*, it was necessary first to exclude piroplasm-induced haemolysis of the collected blood samples (since the pathology of piroplasms is usually associated with haemolysis). The interesting finding was that haemolysis was more significantly caused by inappropriate blood sample handling rather than the haemoparasite itself; and that the higher percentage of haemolysed samples was associated with the Karakamia field site. This highlighted the necessity to review and improve the infield procedures of blood collection in the immediate future.

Once haemolysis by piroplasms was ruled out, possible correlations between AP and the above described haematological parameters were investigated. Surprisingly enough, our study demonstrated a stronger association between AP and Hb only rather than Hb, HCT and RBC count altogether. These three parameters are strictly correlated (Stockham and Scott, 2008). Hb is a more direct measure of blood’s oxygen carrying capacity than HCT and RBC count, but notwithstanding they should finally reflect each other (Stockham and Scott, 2008).
and HCT can be both determined by conductivity methods, which vary in accordance to the analyser used (Stockham and Scott, 2008). Why this finding occurred is yet to be understood completely.
16. Acknowledgements.

This independent study contract could not have been possible without the support and guidance of many people.

I would like to thank my two supervisors, Peter Irwin and Carlo Pacioni from Murdoch University, for the patient, passion and availability, they have demonstrated along the project, and in spite of all the concomitant commitments. Their constant feedback and encouragement has increased my confidence in critical thinking, writing skills and understanding of statistical analysis. Thank you for sharing your knowledge.

I also thank Adrian Wayne from the Western Australia Department of Environment and Conservation (DEC) and Andrew Thompson from Murdoch University, for allowing me to access the Woylie Conservation and Research Project (WCRP) field samples; and to Murdoch University Clinical Pathology Laboratory for the haematology work.

My acknowledgment is addressed to Aileen Elliot from Murdoch University, for the patient she has shown while improving my light microscopy technique.

A sincere thank-you goes to Andrew Smith and Marika Maxwell from DEC, in helping me sorting out the missing data.

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And last but not the least I express my love and gratitude to my parents and my partner, Jeremy Sinclair, who have always trusted my decisions, supported my efforts and transmitted the necessary sense of humour to deal with life.
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18. **Websites.**

Woylie Conservation and Research Project (WCRP)