The population and epidemiological dynamics associated with recent decline of woylies (*Bettongia penicillata*) in Australia.

Carlo Pacioni

DVM, MVS (Cons Med)

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Photo: Sabrina Trocini

To my wife and friend,

Sabrina

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

…….

Carlo Pacioni

Preface

Chapters 5, 6, 7 and 8 are either published papers or manuscripts intended for publication in scientific journals as stand-alone pieces of work. Consequently, some repetition was unavoidable. In addition, some differences in style are due to the requirements of the targeted journal. The reference style of the remaining chapters follows the current guidelines for the journal of *Conservation Biology*.

The intellectual development and writing of this thesis was carried out by the author. Inclusion of co-authors in the papers is to acknowledge the contributions of collaborators who provided tissue samples, demographic data, preliminary analysis and/or background information, as well as helpful discussions and editorial comments.

Abstract

The woylie or brush-tailed bettong (*Bettongia penicillata ogilbyi*) has recently undergone a dramatic decline (approximately 80% between 2001 and 2006). The Woylie Conservation and Research Project (WCRP) was established to investigate possible causes of this decline. It was hypothesised that predators and/or a disease may be a concomitant cause if not the primary cause(s) of the decline, based on the peculiar temporal and spatial characteristics of the decline and available associative evidence.

This research project is an integrated and collaborative component of the WCRP and its broad aim was to contribute to the knowledge on the general health and ecological attributes of woylie populations that were considered directly relevant for the conservation and recovery of the species.

Initially, the WCRP in collaboration with several researchers supported the investigation of specific pathogens. These projects were ongoing when the research described in this thesis began, however there had been no disease risk assessment prior to these ongoing pathogen studies. Therefore, a formal qualitative assessment of the disease risks potentially relevant to the woylie declines was undertaken in this study to ensure a systematic evaluation and to prioritise allocation of resources. Several pathogens were identified as a high priority for further investigation including, but not limited to, Macropod Herpesvirus (MaHV), Macropod Orbivirus (Wallal and Warrego serogroups), and Encephalomyocarditis virus (EMCV).

A haematological investigation was carried out and reference ranges were established. An overall increase of the leukocytic response in animals trapped in Upper Warren (8%, n=23)

compared to woylies in Karakamia (0%) was demonstrated. Gender differences were also recorded, namely males had higher red blood cell, white blood cell and lymphocyte counts than females.

No clear evidence was found that supported an association between changes in the health status of woylies and the decline. Nevertheless, the increased proportion of lymphocytosis ($p<0.0005$) in Perup, which includes two forest blocks that underwent a decline during sample collection, and the higher prevalence of health problems identified during the physical examinations of animals trapped in Upper Warren (41.3%, n=557) as compared to those from Karakamia (10%, n=80, $p<0.0005$. Odds Ratio=6.33, 95% CI 2.99-13.40), justified further disease investigations.

Based on the results of the disease risk assessment and haematological analysis, the serological response to Macropod Herpesvirus (MaHV 1 and 2), Encephalomyocarditis virus (EMCV) and Orbivirus (Wallal and Warrego serogroups) was investigated. There was no serological evidence of any of these viruses affecting woylie populations. Nevertheless, due to sample size limitations, it was not possible to confirm the absence of these diseases with a high level of confidence (i.e. >90%). Additionally, the absence of detection of seropositive individuals does not necessarily imply absence of the pathogen in the population.

Genetic profiles of indigenous (extant wild populations) and translocated woylie populations were examined in order to assess whether woylie populations were suffering from a reduced genetic “health”, as a consequence of the bottleneck that occurred after European settlement. In order to do this a preliminary investigation of the cross-species performance of 32 primer pairs was carried out to assess their suitability for the aims of this study. Twelve microsatellite primer sets were identified as polymorphic and reliable for genetic analysis in woylie.

Additionally, the cross-species performance of the 32 primer pairs was analysed within the potoroines species to facilitate future ecological and genetic studies in bettongs and potoroos. A 50% reduction in amplification success of polymorphic loci for every 1 million years of evolutionary distance from taxa was found and a "priority-list" of markers for use in potoroines was identified.

Genetics does not appear to be a contributing factor to the present woylie decline. Expected heterozygosity (H_E) was around 80%, ranging from 42.3% to 83.6% and the allelic richness (N_{AR}) was around 6, ranging from 2.67 to 9.72. Nevertheless, among the indigenous populations particular concern was raised for woylies at Tutanning Nature Reserve, and for the translocated populations on the South Australian islands. These populations have a substantially reduced genetic diversity (Tutanning: H_E = 0.64; St Peter island: H_E = 0.631; Wedge island: H_E = 0.602; Venus Bay island "A": H_E = 0.423).

Important insights were gained into woylie population structure and dynamics through the analysis of molecular data. Four genetically distinct indigenous populations were identified (i.e. Dryandra woodland and Tutanning Nature Reserve in the wheatbelt region and two discrete populations in the Upper Warren in the south-west forests of Western Australia). The mtDNA analysis showed historical connections between populations in Dryandra and the Upper Warren region (Kingston and Perup). These connections no longer exist as a result of habitat fragmentation caused by agriculture and farming land use. Additionally, substantial gene flow was identified between Kingston and Perup and was supported and quantified by microsatellite analyses in the order of 2-3% migration rate. The evidence of current gene flow within and between populations (i.e. up to 60 km) signifies that direct transmission of an aetiological agent would be possible throughout the whole Upper Warren region within the time frame experienced in the decline.

Analysis of genetic data indicated also that the woylie population in Kingston had already undergone a decline. As a consequence of this change in population abundance, the spatial genetic structure of this population changed, generating a significant correlogram up to 6 km. In other words, in this population, two woylies trapped within a radius of 6 km are likely to be related as opposed to other populations where the genetic signal drops between 1 and 3 km. Additionally, and consistent with previous ecological studies, female philopatry was confirmed and genetic consequences of this behaviour were identified.

Population viability analysis (PVA) demonstrated that the main threatening process for woylie populations is the result of the interaction of various variables (in particular predation and inbreeding) that acquire a considerable strength together, whilst not being greatly significant by themselves. It also quantified the minimum mortality rates necessary for the decline to occur (an average juvenile and subadult mortality rate of 28% and 22% for adults per 91 day time period). The minimum viable population size (MVP) estimated through PVA was consistent with the empirical evaluation based on molecular data (i.e. 1,000-2,000 individuals). As a consequence of the inherent inability of satisfactorily predicting stochastic events and incomplete knowledge on important factors that may affect population size a conservative approach should be adopted. On this basis, a population size of more than 8,000 individuals should be targeted to maximise the likelihood of positive conservation outcomes.

In light of the results of this research project, disease can not be completely dismissed as a possible cause of decline, in particular in association with predation. Haematological, serological and genetic information generated by this study greatly improved the available knowledge on the health and viability of woylie populations and represent baseline data that

will enable monitoring and detection of changes in the health status in these populations, as well as contribute to the refinement of the disease risk assessment and quarantine protocols.

The haematological data will also facilitate and improve the interpretation of disease investigations carried out by the additional collaborative components of the WCRP. Moreover, information obtained on woylie ecology through the analysis of genetic molecular data will assist such interpretations, for example by conveying indications on the frequency and extent of animal movements.

This research also provided suggestions for critical management decisions. For example, the identification of woylie populations at risk of substantial loss of genetic diversity and possibly inbreeding depression calls for appropriate management actions. Where there is no indication of any factor limiting the demographic growth of the populations (i.e. populations on South Australian islands) supplementation was identified as the most suitable management option. Based on the detailed knowledge obtained on the spatial organization of woylie populations, it is now possible to adequately source animals from indigenous populations to augment genetic diversity. Animals should be trapped at a distance of at least 1-3 km in order to maximise the probability that individuals are unrelated. On the other hand, it is critical to identify the causes currently limiting population growth prior to the implementation of these management actions, especially where limited population size (in respect to the carrying capacity) and consequent genetic drift is the main reason for the poor genetic profile of the population rather than isolation.

The PVA also helped to determine critical requirements for the establishment of new, and maintenance of, populations; more specifically that sites should be able to support a minimum

population size of 8,000 individuals and that the average mortality rate should be maintained below 22% for juveniles and 28% for adults per 91 day period.

Finally, this research helped to identify important future areas of investigation. These include longitudinal studies of the health status of individual woylies; epidemiological analysis of the data generated by this study integrated with those generated by other WCRP researchers and a quantification of the influence of ecological factors, such as rainfall and diet, on general health parameters. Additionally, regular genetic monitoring is recommended because the baseline data produced in this study and the associated ecological and demographic data available would provide an optimal opportunity to improve our understanding of genetic consequences of rapid population declines. This monitoring may help to quantify the genetic loss associated with the decline and evaluate the accuracy of PVA predictions. It might be possible to assess the success of management actions (e.g. supplementations) and detect if and when inbreeding depression becomes manifest in populations at lower genetic diversity (e.g. Tutanning).

In addition, the molecular genetic data represents the background work needed to establish the interplay between individual (host) genetic profile and disease susceptibility or fitness (including fecundity and survival). The fact that cross-species primers were used would make the utility of the knowledge acquired easily and directly applicable to other species of the superfamily Macropodoidae.

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Abbreviations

BBN	Balban
BCP	Boycup
CBL	Corbal
CHCM	Corpuscular haemoglobin concentration mean
CHP	Chariup
CMR	Camelar
DEC	Department of Environment and Conservation
EMCV	Encephalomyocarditis virus
FBG	Fibrinogen
HDW	Haemoglobin distribution width
HGB	Haemoglobin concentration
KAR	Karakamia
KING	Kingston
KNP	Keninup
MaHV	macropod herpes virus
MCH	Mean corpuscular haemoglobin
MCV	Mean cell volume
MPN	Moopinup
PCV	Packed cell volume
PER	Perup
PVA	population viability analysis
RBC	Red blood cells
RDW	Red cell distribution width

r_{mt}	Mantel test correlation coefficient
r_{SAc}	Spatial autocorrelation correlation coefficient
SA_C	Spatial autocorrelation
TP	Total protein
VNT	Virus neutralization test
VNTR	Variable Number Tandem Repeat
WBC	Total white blood cells
WCRP	Woylie Conservation Research Project
WDRC	Woylie Disease Reference Council
WJP	Winnejup
WRP	Warrup

1 General introduction

1.1 Introduction

Global biodiversity and more specifically wildlife conservation issues are receiving increasingly broader and more popular attention. The rate at which extinction is occurring in wildlife species is now faster than what would be expected by the effect of natural selection alone (Pimm & Raven 2000). In Australia, in particular, mammal extinctions and declines in the last 200 years have occurred at an unprecedented rate (McKenzie et al. 2007) and anthropogenic changes are primarily responsible for the extinctions observed around the world and in Australia (Rosser & Mainka 2002). Active management is often needed in order to reverse the decline of an endangered species (McKenzie et al. 2007). As a result, promoting changes to human activities and behaviour are a necessary step to promote biodiversity conservation (Brandon 2001).

Identifying the cause of a wildlife population decline is a critical and crucial step for ensuring that effective management actions are undertaken (Caughley 1994; Caughley & Gunn 1996). However, identifying the main cause of decline is very challenging and often impossible, because typically, multiple factors are responsible for a species' demise, and these factors are often entangled in a complex matrix of confounding variables (Caughley 1994; Caughley & Gunn 1996; Peery et al. 2004). Also, crucial baseline data regarding endangered species (such as demographic dynamics or health parameters) are generally either not available or inadequate. Therefore, a systematic approach is necessary to assess potential causes of decline in wildlife (Caughley 1994; Caughley & Gunn 1996). Retrieving critical information on the species' ecology and indirect evidence of potential causes of the decline have been

indicated, among others, as fundamental components of this approach (Caughley 1994; Caughley & Gunn 1996).

This research project was established based on considerations of these principles and it is hoped that it will represent a contribution towards woylie (*Bettongia penicillata ogilbyi*) and, possibly, Australian native fauna conservation. The woylie, also called brush-tailed bettong, is an iconic marsupial in that it was the first species to be removed from 'Listed Species' in the Commonwealth Endangered Species Protection Act (Start et al. 1998). Nevertheless, woylies are now undergoing a dramatic decline (Groom 2010), which prompted a review of the species' conservation status (Wayne et al. 2009) and an investigation of the possible causes of decline. This chapter reviews the main biological and ecological features and the demographic history of this species that are relevant to this research project. The study aims and thesis structure are also outlined.

1.2 Biology and ecology

Woylies are small macropods belonging to the subfamily of potoroines (Westerman et al. 2004). Their body length is between 300 and 360 mm, with the weight ranging between 750-1500 g. Woylies do not show evident sexual dimorphism, however males can be slightly heavier and reach 1850 g (Van Dyck et al. 2008).

The woylie diet mainly comprises hypogeal fungi (native truffles) although they can also eat tubers, bulbs, seeds and invertebrates (Van Dyck et al. 2008; K. Zosky[1], personal

[1] Kerry Zosky. Unpublished. Food resources and woylie dcline in south-western Australia. Murdoch University, PhD thesis.

communication). They play an important ecological function. For example, woylie diggings improve significantly the soil turnover and water infiltration (Garkaklis et al. 2000, 2004), while their seed caching habits are important for sandalwood regeneration and distribution (Murphy et al. 2005).

Females reach sexual maturity at 6-8 months and males at 10-12 months (Sampson 1971; Christensen 1980). Normally, females give birth to only one young at a time after a gestation of around 21 days (Smith 1992). While twins have been reported, the probability of survival of the second young is believed to be limited (Sampson 1971). Indigenous (continuously persistent) populations show continuous reproduction throughout the year (Sampson 1971; Christensen 1980; Ward et al. 2008a) with a birth interval of approximately 100 days (Sampson 1971; Christensen 1980). Consequently, females can give birth to up to three young per year. However, evidence of a breeding season was detected in Karakamia – a fenced, translocated population, near Perth (Ward et al. 2008a) – suggesting that food resources may influence woylie reproduction capacity. Similar to other macropods, the woylie has embryonic diapause (Tyndale-Biscoe 1984). After mating, which induces ovulation, the blastocyst becomes quiescent and will reactivate its growth only once the pouch is empty again, reaching its full development within 17.5 days (Smith 1992).

The home range of woylies includes a nesting area and a feeding area. Nests are usually built above a shallow dig using leaves, grass, or other objects found in the vicinity that are transported by curling the tail around the nesting material. The woylie is usually highly protective of the nesting area and animals do not share nests in the wild (Sampson 1971; Christensen 1980), while sharing of nests has been reported between females and non-breeding males in captivity (Delroy et al. 1986). On the other hand, they commonly share feeding areas. Average size of the home range was found to change slightly depending on the

method used to calculate it, habitat attributes and gender – with males considered to be more mobile than females – but it was consistently within the range of 19.6-34.8 ha (Sampson 1971; Christensen 1980). Woylies increase their home range through inspections of contiguous areas, but no substantial changes are expected over a short period of time (Christensen 1980).

Dispersal is believed to occur before sexual maturity is reached, with female survival after dispersal being higher than males (Sampson 1971; Christensen 1980). Once the animal settles in a new territory, there is no evidence of long distance movement, even during bush fires. Christensen (1980) recorded only a few animals moving away from established territories within areas that were subsequently burnt. When they did so individuals typically moved no further than to the edge of the burnt area. However, woylies are capable of moving relatively long distances (3-5 km) as shown by Christensen's (1980) transportation experiment and observations of movement subsequent to a translocations (Martin et al. 2006).

1.3 Distribution and post-European decline

The woylie was distributed across much of Australia until the arrival of European (Figure 1.1). By the 1970's their range in the wild was reduced to only three Western Australian locations: Dryandra woodland (Dryandra), Tutanning Nature Reserve (Tutanning) and Upper Warren (Figure 1.2).

Fox (*Vulpes vulpes*) predation was thought to be the main cause of woylie declines in the 19th and 20th centuries (Christensen 1980; Burbidge & McKenzie 1989; Start et al. 1998). This hypothesis was also supported by the fact that woylie populations showed a positive response to predator control activities, especially after the establishment of the Western Shield program

(Start et al. 1998; Mawson 2004; Orell 2004). Despite the fact that this seems to be the case for most locations, Sampson (1971) did not find any evidence of predation being important in Tutanning, while habitat quality appeared to be the most critical factor influencing woylie density and distribution within the reserve. According to Sampson (1971), the woylie avoids open areas that lack understorey, preferring thick scrub that provides protection for both foraging and shelter. Sampson (1971) was able also to demonstrate that high quality nesting areas are essential for the conservation of this species because not only do they provide protection from predators, but they also provide a critical shelter from hot weather, reducing water requirements especially during droughts. Habitat loss through land clearing for farming has also been a significant factor in the species' range contractions (Van Dyck et al. 2008).

In 1996, woylies were the first species to be removed from 'Listed Species' in the Commonwealth Endangered Species Protection Act and Western Australia State Government Wildlife Conservation Act 1950 (Start et al. 1998) as a consequence of the positive response of woylie populations to the predator baiting program and associated translocations.

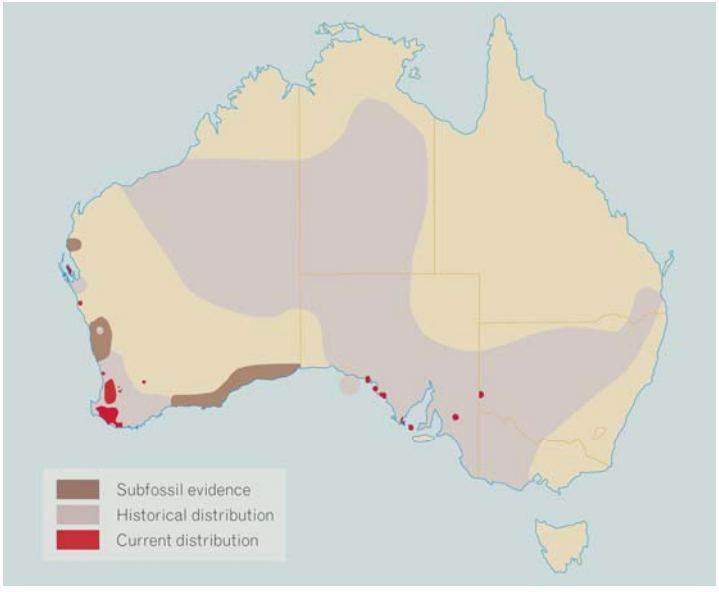

Figure 1.1 Woylie past and present distribution (Source: Wayne et al. 2008b).

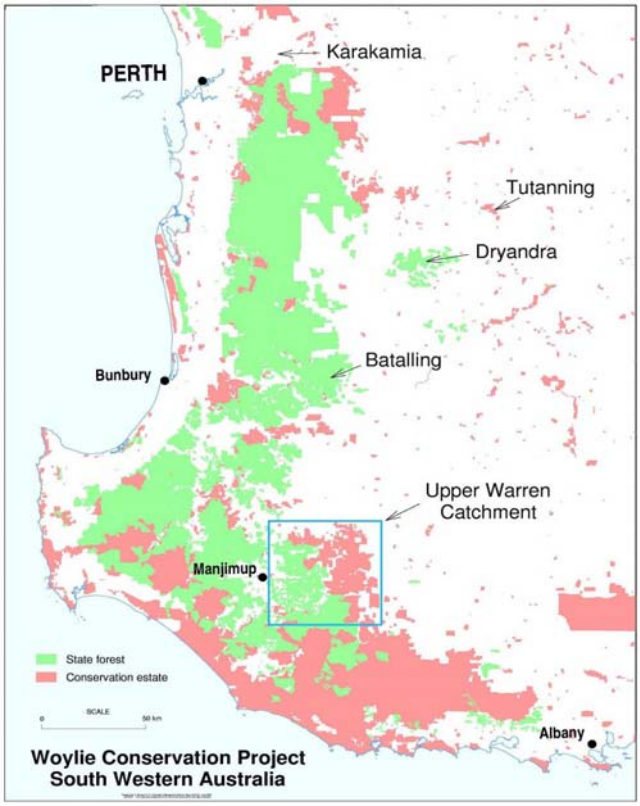

Figure 1.2 The location of important woylie populations in Western Australia (Source: Wayne 2008).

1.4 Translocations

In comparison with other single-species management plans, an unusually high number of translocations have been carried out for the woylie (e.g. Mawson 2004; Finlayson et al. 2010) due to a combination of factors which include their high reproductive rates, initial abundance of the source populations and their adaptability to a variety of climatic and habitat conditions. The history of these translocations is briefly summarised only for the populations relevant for this study (Table 1.1).

Batalling State Forest (Batalling): A total of 52 woylies were moved from Chariup (Upper Warren) in 1982 and 1983 to Batalling (Figure 1.2), a site east of Collie (Start et al. 1995). Similar to the naturally occurring populations, Batalling showed an increasing population size proportional to the predator control effort. Woylies were at undetectable levels until 1991 when regular baiting of a section of the area started (Orell 2004). Baiting was then expanded under the "Operation Foxglove" and by 1996, the woylie capture rates were so high (around 40%) that it was difficult to monitor other species using conventional trapping methods (Orell 2004; Wayne et al. 2008a).

Karakamia Sanctuary (Karakamia): Karakamia was established in 1992 and received translocated woylies in 1994, and then again in 1995 and 1996, with a total of 31 woylies released into the sanctuary. These animals mainly represented Dryandra genetic stock, although two animals were sourced from Upper Warren. Additional supplementations are being carried out occasionally using animals from rehabilitation centres (J. Williams[2], personal communication). Karakamia increased its land-area over the years, with adjacent private land being added to the sanctuary to reach the current size of 275 ha, and is completely fenced. Although some parts of the sanctuary were previously used for grazing and Jarrah timber-harvesting, most of the area retains native vegetation or has been revegetated. The estimated overall carrying capacity of the sanctuary is around 400-600 individuals (J. Williams, personal communication). In more recent years, due to its high density and stable population, Karakamia has been used to source woylies for subsequent translocations (Mawson 2004). In fact, almost every year after 2000, between 51 and 162 individuals were translocated from this population (J. Williams, personal communications).

[2] Williams, J. Field Ecologist - Australian Wildlife Conservancy.

South Australia (SA): Three translocated populations from SA have been included in this study: St Peter Island, Wedge Island and Venus Bay Island A (Figure 7.1). All of these islands are free from feral predators. A breeding program was established to provide a source of woylies for translocation to the SA islands (Delroy et al. 1986). Only three woylies (two females and one male) were used as founders of the breeding program and these were made available by Perth Zoo and George Wildlife Reserve, which also originally obtained their animals from the Perth Zoo (Delroy et al. 1986). The origin of Perth Zoo's stock remains unclear (Delroy et al. 1986).

St Peter Island (StPI): This is the largest of the three islands. A total of 113 woylies were released in 1989 (Nelson et al. 1992). After preliminary genetic screening that showed a low level of VNTR (Variable Number Tandem Repeat) genetic diversity – 80% band sharing (Start & Armstrong 1994; Start et al. 1998) – a "boost" translocation was attempted in order to improve the genetic variability of this population. An additional 15 woylies (ten males, five females) from Dryandra were released in 1996, but unfortunately only six (four males, two females) were found in good condition after only two months following the release (Van Weenen 1996), consequently the extent of their genetic contribution remains unknown.

Wedge Island (WEDI): It is the second largest of the three islands. Only woylies were released in 1983 (Delroy et al. 1986) and became quickly established (Nelson et al. 1992). Similar to StPI, preliminary genetic assessment indicated low levels of genetic diversity (Start & Armstrong 1994; Start et al. 1998) and two attempts were made to increase the genetic variability: one moving 10 males in 1994 (Start & Armstrong 1994; Van Weenen 1996), and the second introducing 15 males in 1995 (Van Weenen 1996) but both failed with most animals found dead or not re-trapped in subsequent monitoring (Start & Armstrong 1994; Van Weenen 1996).

Venus Bay Island A (VBIA): This is a very small island of only 15 ha. Seven woylies were released in 1980 (Delroy et al. 1986). By 1984 more than 53 individuals were present on the island (Delroy et al. 1986). However, the population size stabilized at around 30 animals as a result of the limited carrying capacity of the island (Start 1993).

Table 1.1 Main woylie locations, area and total number of released animals

Locations	State	Area (ha)	TNR
Upper Warren	WA	236,936^	NA
Dryandra Woodland	WA	12,192	NA
Tutanning Nature Reserve	WA	2,369	NA
Batalling State Forest	WA	3,617^	52[1]
Karakamia Sanctuary	WA	275	31[2]
St Peter Island	SA	3,493	113[*3]
Wedge Island	SA	947	11[*4]
Venus Bay Island	SA	15	7[4]

TNR: Total Number Released. **NA**: wild population, therefore not applicable. ^ approximate extent of occurrence, forest habitat is contiguous across a much larger area (i.e. not isolated and as discretely defined as wheatbelt and island populations). ***** Additional releases have been carried out (Van Weenen 1996). [1](Start et al. 1995); [2] J. Williams, personal communication; [3] (Nelson et al. 1992); [4] (Delroy et al. 1986).

1.5 The current decline

With the exception of the populations maintained within enclosed predator-proof sanctuaries managed by the Australian Wildlife Conservancy, recent declines have threatened all high-density populations in Western Australia and the mainland population in South Australia (Wayne 2006; DEC Science Division 2008a). Currently, declines have been between 93% and 97% in Western Australian populations and 90% in Venus Bay Conservation Park, in South Australia (Armstrong 2008; Swinburn et al. 2008; Ward et al. 2008a).

The declines were almost synchronised throughout Western Australia. First signs of decline in Dryandra were detected in 2001 (Swinburn et al. 2008; Wayne 2008), while demographic data showed that in Batalling and Upper Warren the decline started between 1999 and 2002 (Wayne 2006; Swinburn et al. 2008; Wayne 2008).

In response to the massive reduction in numbers in such a short period of time, woylies were re-listed as 'Critically Endangered' in the IUCN Redlist (Wayne et al. 2009), and the Department of Environment and Conservation (DEC) established the Woylie Conservation and Research Project (WCRP), led by Dr Adrian F. Wayne, to investigate possible causes of decline (http://www.dec.wa.gov.au/content/view/3230/1630/).

In Upper Warren, demographic data allowed the demonstration that the decline followed a specific spatial pattern. Southern blocks in the Perup (eastern portion of the Upper Warren; Figure 1.3) were the first to be affected by the decline, which then progressed north at a rate of 5-10 km per year, leaving Keninup, the most northerly block, as the last location with medium density (Figure 1.4).

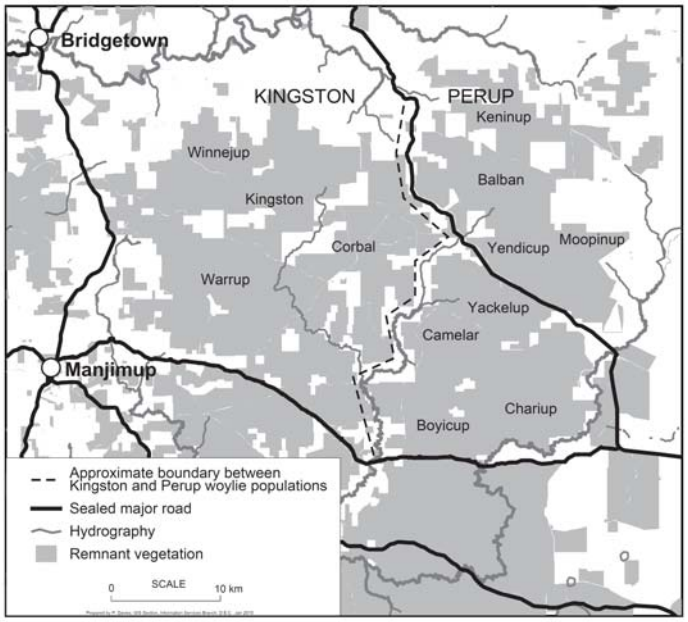

Figure 1.3 Woylie populations and sampling sites within the Upper Warren region.

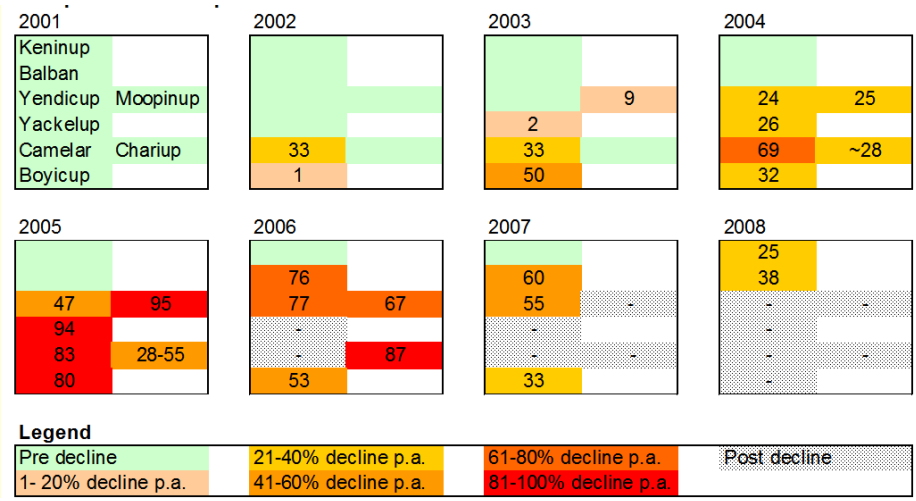

Figure 1.4 Schematic representation of the spatial declines of the woylie in Perup Nature Reserve, Upper Warren (Source: A. Wayne[3] unpublished data).

Chariup rate of decline for 2004 and 2005 is estimated since no data were available.

[3] Adrian F. Wayne. Research Scientist (Forest Fauna Ecology) Science Division, Department of Environment and Conservation, Western Australia.

The temporal characteristics of the decline were also quite peculiar and similar dynamics between forest blocks were evident. For example, little difference was found between the yearly rates of decline over time among forest blocks in Perup Nature Reserve (eastern sites of Upper Warren) (Figure 1.5; Wayne et al. 2008b). Pre-decline capture rates were always high (i.e. the decline always started when density was high: more than 40%, average 62%). The decline progressed over a period of 4-5 years in each forest block and when the density reached a low level (less than 10%) it has apparently stabilised (Ward et al. 2008a; Wayne et al. 2008b). As an example, capture rates over time in the Perup Nature Reserve are presented in Figure 1.6.

Given the characteristics and demographics of the woylie decline, Wayne et al (2006) hypothesised that while predation by introduced predators played an important role, a disease may be a concomitant cause if not the primary cause of the decline. Details of population dynamics associated with these recent woylie declines are described in depth elsewhere (Wayne et al. in preparation).

Given the possibility of a disease being responsible for the decline, specific expertises in disease investigation were sought and the Woylie Disease Reference Council (WDRC) was established in 2006. The WDRC is an interdisciplinary forum that brought together disease experts from Murdoch University and Perth Zoo providing support and scientific advice for research investigation of specific pathogens.

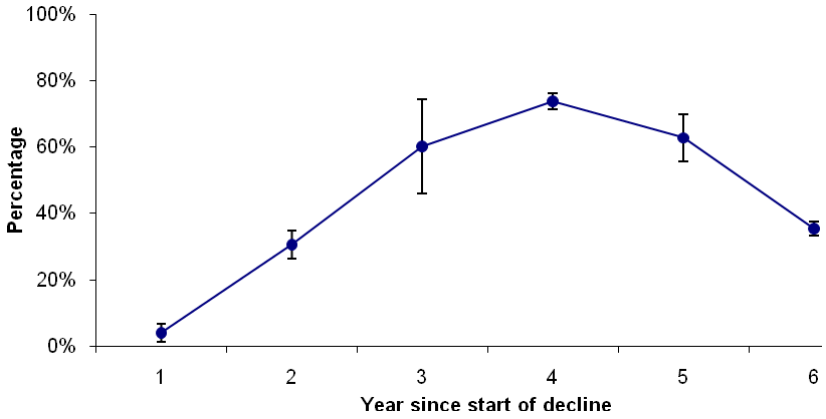

Figure 1.5 Average rate of decline (percentage, error bars: one standard deviation) in woylie capture rates of different forest blocks in Perup Nature Reserve adjusted to year since the start of decline (Source: Wayne et al. 2008b).

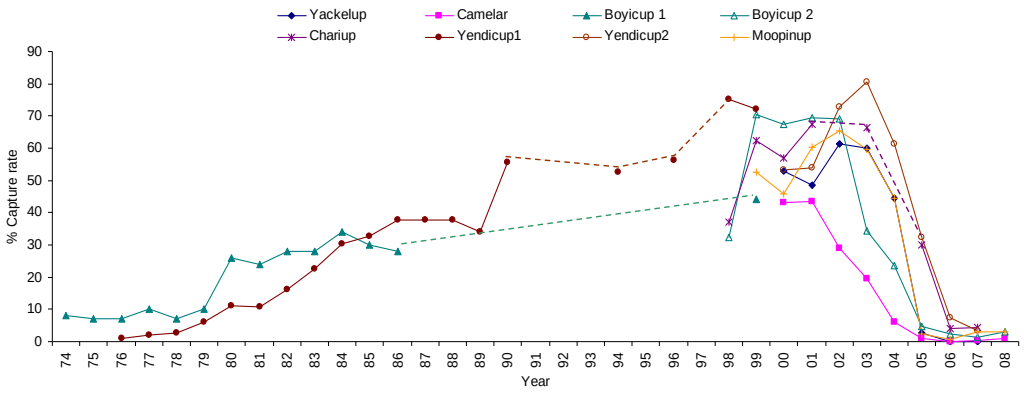

Figure 1.6 Capture rates of woylies over time in Perup Nature Reserve (Source: Wayne et al. 2008b).

Note: Transect names with the suffix 1 and 2 distinguish relatively similar transects within the same area with slightly different methodologies. The dashed lines are indicative trends during the intervening periods between trapping events in non-successive years. See Figure 1.3 for geographic location of sampling sites.

1.6 Study aims and thesis structure

This research project was an integrated and collaborative component of the WCRP and its broad aim was to contribute to the knowledge on the general health and ecological attributes of woylie populations that were considered directly relevant for the conservation and recovery of the species. In particular, specific variables that could be responsible for, or indicate a change of, the status of health were considered including physical condition, haematology, genetic profiles and presence of specific pathogens. Ecological features that were considered to be directly relevant to the assessment of the risks of transmission of diseases and to improve the understanding of the decline, as well as the capacity of woylie populations to recover included: spatial organisation of woylie populations, dispersal modalities and population dynamics in response to different factors such as different levels of mortality rates, genetic inbreeding and feral predation.

The WDRC had already been established and several studies that intended to investigate the presence of specific pathogens were ongoing, when this research project began. Nevertheless, a formal assessment of the disease risks that could be potentially relevant in relation to woylie declines had not been undertaken. In Chapter 2 the theoretical approach used to enable the selection of additional disease investigations that should be included in the WCRP is described. Pathogens that were already under investigation by other researchers were not taken into consideration in this PhD research program.

Chapter 3 provides baseline data on the haematological panel in woylies that forms the basis for the diagnosis and identification of sick animals. Furthermore, it was evaluated whether changes in the general health of individuals (haematological and physical condition) were associated with the decline.

In Chapter 4, the presence of selected diseases ranked as a high priority (see Chapter 2) was investigated. Time and cost limitations constrained the number of diseases that could be investigated and not all of the high priority diseases were examined.

It was explored whether the genetic diversity of woylie populations (indigenous, Chapter 6 and translocated, Chapter 7) was reduced and could potentially be responsible for an overall reduction of fitness (e.g. inbreeding depression). Historical information reported that the woylie underwent the typical conditions of a bottle neck effect (since the population collapsed and then partially recovered). A possible outcome of this well-known phenomenon would be decreased genetic diversity and therefore the likelihood of decreased capacity of the population to cope with changes in the environment and/or other challenges, with a consequent substantial impact on the survivorship of the populations. In this context, the genetic profiles of populations were evaluated as a health risk factor. In order to do this a preliminary investigation of performance of cross-species primers was carried out in order to assess their suitability for the aims of this study (Chapter 5). Additionally, population relationships, both historical and modern, were investigated, identifying and quantifying long distance movements and the associated risks of disease transmission (Chapters 6 and 8). Finally, management actions that would contribute to the conservation of the species, in relation to the preservation of their genetic diversity, were suggested (Chapters 6, 7 and 8).

In Chapter 8 analyses the genetic structure and spatial distribution of genotypes of woylies in the Upper Warren populations is analysed and these attributes compared to the other two indigenous populations (Tutanning and Dryandra) to provide insight into woylie dispersal characteristics. Knowledge about these features is important because it not only improves the understanding of the ecology of the species, but also could be helpful in defining the

epidemiology of diseases that are potentially threatening declining populations. A molecular based assessment of demographic changes of the populations whose complete demographic data were not available was also carried out and is presented in this chapter.

An individual-based model was developed to describe population dynamics and analyse the modalities of the responses of woylie populations (Population Viability Analysis, PVA). In particular, this model was used to quantify the maximum "sustainable" mortality rates (i.e. the maximum level of mortality that could be sustained by a population, beyond which extinction would become a highly likely event). These estimates are considered to be an indirect indication of the minimum mortality rates that were necessary for the decline to occur. Using data obtained from the genetic analyses, the potential role of genetic inbreeding in the long term conservation of woylie populations and the synergistic effect between one factor reducing fitness and a second causing death were also explored (Chapter 9).

Finally, a synthesis of the main findings of this project and their significance in terms of woylie conservation is provided (Chapter 10). Potential future research directions are also discussed.

2 Identification of potential diseases and qualitative risk assessment in relation to recent woylie declines.

Most of the work described in this chapter was included in the progress report of the woylie conservation research project "Diagnosis of recent woylie (Bettongia penicillata ogilbyi) declines in south-western Australia. A report to the Department of Environment and Conservation Corporate Executive" (DEC Science Division 2008a).

2.1 Introduction

The role of disease as a contributing or determining factor in wildlife decline or extinction is being increasingly recognised (Spalding & Forrester 1993; Daszak & Cunningham 1999; Daszak et al. 2000; Spielman 2001). In fact, it has been suggested that disease screening should be included in wildlife monitoring programs and conservation projects (Spalding & Forrester 1993; Aguirre et al. 2002).

A significant challenge for this process is the paucity of known information on diseases in specific wildlife species (Wobeser 2007) and often the complete absence of baseline data to determine what could be considered as "normal" (Spalding & Forrester 1993). Additional

complications may arise due to financial constraints and practical limitations. Moreover, diagnostic techniques are not always available or validated for the species under investigation and use of tests developed for other species is not always reliable (Wobeser 2007). Therefore, it is necessary to use a logical and scientifically based process to address these limitations and develop a realistic solution that provides a comprehensive view of these important issues.

Based on disease risk assessment models proposed in the literature, especially for wildlife translocations, this study utilised a systematic approach in order to determine which diseases should be prioritised for further investigation (Spalding & Forrester 1993; Jakob-Hoff et al. 2001; Munson & Karesh 2002; Armstrong et al. 2003; Miller 2007).

2.2 Methods

Since a quantitative evaluation of the disease risk was not achievable due to a lack of knowledge of disease prevalence and species susceptibility, a qualitative approach was used. Jakob-Hoff et al. (2001) showed that a qualitative disease risk analysis was a practical and useful tool to achieve goals similar to those of this investigation. A semi-quantitative approach has been also used in similar circumstances (e.g. Murayama et al. 2006 in Miller 2007). However, because an explicitly descriptive assessment is recommended for this type of investigation (Armstrong et al. 2003; Miller 2007; Watson et al. 2007), it was decided that a qualitative method would be more flexible and appropriate. As suggested (Munson & Karesh 2002; Armstrong et al. 2003), important diseases reported to have occurred in bettongs and other sympatrically occurring macropods were considered. This was accomplished through a broad literature review and the acquisition of unpublished critical data from the Australian Wildlife Health Network (AWHN).

Investigations into haemoparasites, endoparasites, ectoparasites, *Toxoplasma gondii*, and *Salmonella* spp. were specifically investigated by the WCRP (Clark & Spencer 2007; Abdad et al. 2008; Burmej et al. 2008; Parameswaran 2008; Parker et al. 2008; Smith et al. 2008a) and will not be discussed further here. The purpose of this qualitative disease risk analysis was to identify additional potential diseases of significance for future investigation by the WCRP.

Initially a list of diseases that were considered to be important was generated and following Armstrong et al. (2003), specific criteria were used to rank the importance of a given disease, including:

- Geographic distribution of the pathogens
- Species reported to be affected by the disease
- Known presence of the disease (in captive and wild populations)
- Mode of transmission of disease
- Outcome of infection (e.g. clinical signs, pathologic lesions)
- Virulence of the disease (on its own accord or in synergy with other pathogens)

A qualitative grading system was applied to these factors to determine the significance of the disease in relation to population declines, and therefore assist in guiding future directions for disease investigations in the WCRP.

Based on the known geographic distribution and the documented presence in woylies or related species, the *likely presence* of pathogens in wild woylie populations (see Tables 2.1-2.3) was ranked (scored) as *low*, *moderate* or *high*. When a disease was detected only in captive populations, it was estimated, from the available information, whether the captive circumstances may have altered the epidemiology of the disease and the final score for the *likely presence* in wild populations was adjusted accordingly. For example, aspiration pneumonia caused by *Pseudomonas* spp. in joeys was reported only in hand reared individuals.

Although this condition could go undetected in the wild, it was considered that the captive condition was likely to increase the onset of this pathology (e.g. forced-feeding). Consequently, the *likely presence* score was "low" in spite of the fact that woylies may be affected.

The *independent* (i.e. by itself) and *in concert* (i.e. in association with other pathogens) pathogenic potential (see Tables 2.1-2.3) was estimated as *low*, *moderate* or *high* considering the mode of transmission and outcome of infections. The potential disease would be expected to cause a demographic decline of a similar entity as documented by trapping data (Wayne et al. 2008b; Wayne et al. 2008c). Based on field data, fertility rates were not affected during the decline and dispersal was discounted as a cause of reduced re-trap rate of adults; therefore, mortality was deduced to be responsible for the reduced densities of and this was confirmed with the radio-tracked cohorts (Wayne 2006; Ward et al. 2008b). Given that mortality was considered the cause of the population decline (Wayne 2006), only diseases capable of causing high and widespread mortality or debilitation of animals up to a stage where death would arise due to secondary causes (e.g. predation) received high scores.

The final *risk/priority* score was generated by integrating the above parameters in relation to population declines. Field data collected in the preliminary stage (2006-2007) of the WCRP indicated that the potential disease was likely to have an acute onset. It should be noted that an analysis of field health examinations and haematology data had not been carried out at this stage (Chapter 3) and only general summaries were available from the DEC. However, it was understood from DEC researchers that the overall body condition scores were high before the decline and no significant decrease could be detected during or after the decline. Consequently, diseases associated with chronic illness were discounted. For instance, despite a moderate score in the *likely presence*, *independent* and *in concert* pathogenic potential categories, infection from *Mycobacterium* spp. received a low final *risk/priority* score because

it was judged that, due to the chronic nature of this disease, general symptoms such as decreasing body condition in woylies would have been previously detected.

Following completion of the disease risk analysis, the results were presented to the WDRC and were discussed in a consultative and multidisciplinary setting.

2.3 Results

The list of selected disease agents considered to potentially affect woylies is summarised in Tables 2.1-2.3. These pathogens were considered to have the potential to cause rapid and widespread population declines based on the outcomes of this risk assessment process.

Diseases considered of significance, which will require future investigation by the WCRP included:

- Chlamydiales
- Macropod Herpesvirus
- Macropod Orbivirus
- Encephalomyocarditis virus
- *Neospora caninum*

Arboviruses have been reported to occur in macropods associated with subclinical infection (Russell 2002; Old & Deane 2005). These viruses are potentially zoonotic and therefore may also warrant further investigation.

Certain disease agents were ranked as having a low or very low priority because they were rarely reported to occur or had not previously been identified in Western Australia. These included: *Clostridium tetani*, *Leptospira interrogans*, *Pseudomonas pseudomallei*, *Coxiella burnetti*, *Rickettsia rickettsii*, *Leishmania* spp., Adenoviruses and 'wobbly possum syndrome'. A

number of other pathogens were considered to be opportunistic or secondary infections and unlikely to be responsible for population declines in their own right. These included: *Pseudomonas* spp., *Escherichia coli*, *Klebsiella* spp. and *Clostridium piliforme* (Tyzzer's disease). Other diseases, such as Lumpy jaw, Mycobacteriosis, and a number of fungal diseases, were considered chronic diseases that would likely result in different decline patterns and observations than those seen in the woylie decline (Ward et al. 2008b).

2.4 Discussion

Of the diseases considered important for further investigations identified by this risk analysis process, Macropod Orbivirus infection was considered a high priority. Orbivirus infection, especially by the Wallal and Warrego serogroups, have been reported to infect several species of macropods that are closely related to woylies, with serious clinical consequences (see Table 2.2 for details) (Daszak et al. 2000). Therefore, woylies may be potentially susceptible to this viral pathogen. At its extreme, a severe clinical form, similar to that described for other kangaroos (e.g. western grey kangaroos, *Macropus fuliginosus*), could have critical consequences in woylie populations. A number of epidemics of this disease have been reported in wild populations of macropods in Australia between 1969 and 1996, including outbreaks in the South West region of Western Australia (Hooper 1999; Hooper et al. 1999). Hooper et al. (1999) also reported a high seroprevalence of these viruses in wild kangaroos and wallabies throughout the region. The virus was isolated from eye and brain tissues collected from kangaroos near Albany, Esperance and Perth (Hooper et al. 1999). Consequently, it can be concluded that the virus is well established in the region.

Macropod Herpesvirus (MaHV) is another viral infection that is present in the region. MaHV has resulted in sudden death in captive populations of grey dorcopsis wallabies (*Dorcopsis*

muelleri luctuosa), quokkas (*Setonix brachyurus*), western grey kangaroos and woylies (Dickson et al. 1980; Callinan & Kefford 1981; Wilks et al. 1981). Antibodies have been found in both wild and captive populations of macropods. A study of antibody levels against MaHV indicated a higher prevalence of antibody in captive populations, with the highest levels found in a captive population of tammar wallabies (*Macropus eugenii*)experiencing an outbreak of MaHV (Webber & Whalley 1978). High antibody levels in captive animals may reflect a higher level of virus transmission due to crowding, increased contact rates with infected animals, or increased stress leading to expression of latent virus (Webber & Whalley 1978). The fact that woylie populations started declining when they reached the highest density levels would suit the ecology of this infection. However, the widespread distribution of antibody titres in marsupials, as discussed by Webber and Whalley (1978), suggests that this virus has evolved with marsupial species and may be endemic in wild populations. Herpes viruses can, in some instances produce severe acute disease with mortality, especially in young animals and with some herpes viruses after introduction into a new species (e.g. Aujeszky's disease virus, herpes B virus of macaques, alcephaline herpes virus 1) (Murphy et al. 1999). Dickson et al. (1980) reported an outbreak of MaHV infection that resulted in the sudden death of eight woylies, among other species, over a period of a week. This report indicates that woylies may be highly susceptible to MaHV infection, although with opportunistic surveillance as conducted with wild woylie populations, it could be difficult to detect the presence of MaHV disease in a population. Clinical symptoms, such as ulcerations or vesicles, may be rare and difficult to detect in field investigations. In this scenario, an affected population could undergo a steep decline, with increased mortality after introduction of the virus, similar to what the available monitoring data suggests. Further investigation into this disease as a potential aetiological agent of population decline, would initially require examination of the woylie serum bank collection for serological evidence of MaHV infection.

Chlamydiales are bacteria known to cause significant disease in some marsupial species. For example, they can cause conjunctivitis and proliferation of the eyelid in western barred bandicoots (*Perameles bouganville*) and greater gliders (*Petauroides volans*) (Bodetti et al. 2003), and conjunctivitis, keratitis, cystitis as well as infertility in koalas (Holz 2003). In addition, *Chlamydiales* types are known to be present in the south-west of Western Australia because they have been isolated from a number of marsupial species in this region such as western barred bandicoots and greater bilbies (*Macrotis lagotis*) from Dryandra, and Gilberts' potoroo (*Potorous gilbertii*) from Albany (Bodetti et al. 2003). However, the clinical significance in macropods, and especially the possible role of this pathogen in the woylie decline, is still unclear. The lack of information about this pathogen in the affected woylie populations is a significant gap in determining the clinical importance of these agents.

Encephalomyocarditis virus (EMCV) is a known pathogen in rural areas in Australia and it can occur when rodents build up to plague proportions around piggeries. The disease in pigs is acute with sudden death or acute neurological signs. EMCV was isolated from a variety of Australian native fauna, including macropods (*Macropus rufogriseus*, *M. rufus* and *Dendrolagus goodfellowi*), causing sudden death as the only sign (Reddacliff et al. 1997). More recently, a case of sudden death caused by EMCV was reported in a Lumholtz's Tree Kangaroo (*Dendrolagus lumholtzi*) (David Blyde[4], personal communication), as well as an infection in a quokka (McLelland et al. 2001). The variety of hosts and the seriousness of the consequences of infection resulted in the evaluation of immune response of captive colonies of eastern wallaroos (also called euros, *Macropus robustus*) to an EMCV vaccine (McLelland et al. 2005). Seen the success of the vaccination trial, the author recommended its use in non-domestic species. If woylies are susceptible to EMCV, it could have serious consequences for the species. Therefore, it is recommended that woylie sera be tested for exposure to this virus.

[4] David Blyde. Veterinarian, Sea World.

Neospora caninum is a recently discovered (first identified in 1984) protozoa (Bjerkås et al. in Reichel 2000). It is now recognised to be world-wide and has been isolated from a variety of species including dogs, cattle, sheep, goats, deer and horses (Reichel 2000). Its clinical significance in wildlife is not yet understood, but considering the seriousness of the clinical signs (paresis or paralysis in dogs and abortion in cattle), it was considered worthwhile to further investigate the possible prevalence of this infection in woylie populations. It may be possible to adapt existing serological tests for this parasite for use in woylies.

Arboviruses are mosquito-born viruses comprised of Flaviviruses (Murray Valley Encephalitis and Kunjin present in northern Western Australia) and Alphaviruses (Ross River Virus and Barmah Forrest Virus present in Western Australia). Some of the infections caused by these viruses are important zoonoses and they should be given special consideration. Macropods are known to have been infected with the Flaviviruses and Alphaviruses but have not shown any evidence of disease and sero-epidemiological studies suggest they could be possible virus reservoirs (Russell 2002; Old & Deane 2005). Although it is not expected that either of these viral genera would be causing disease problems in woylies, knowing whether this species of macropods is contributing to maintain the virus in the habitat would be of great interest in terms of human health. Furthermore, a positive serological result could give an indication of the exposure of this species to biological vectors such as mosquitoes.

Two other viruses affecting European rabbits (*Oryctolagus cuniculus*) that are present in Western Australia, Rabbit Calicivirus and Myxomatosis virus, were also considered in relation to possible effects on woylies and were discounted. Both Rabbit Calicivirus and Myxomatosis viruses are very host specific; had been evaluated in a wide range of Australian wildlife before

use for biological control of European rabbits and since release have not been found to infect animals other than rabbits (Ratcliffe et al. 1952; Bureau of Resource Sciences 1996).

Unfortunately, it was not possible to include toxicoses in the risk assessment. Due to time and financial limitations, as well as a lack of expertise within the WDRC in this field, the potential role of toxicoses in the woylie decline was not explored. It is acknowledged that this is a serious limitation of this initial study and recommendations were made to the WDRC to facilitate the creation of a dedicated team to undertake this important task. Environmental pollution and disruption of established ecosystem equilibria can have dramatic consequences for wildlife populations, as well as human health (e.g. Burkholder 2002; Aguirre & Tabor 2008). It is well known, for example, the role that the heavy use of pesticides had in the decline of raptor populations worldwide (Anderson et al. 1969; Stickel et al. 1984; Newton 1988). Land use for agriculture and livestock in the areas surrounding most of the localities where woylies occur nowadays represents a likely source of environmental pollution and the possibility of toxicoses occurring in woylie populations should not be discounted.

2.5 Conclusion

A qualitative approach, as suggested by the literature (Jakob-Hoff et al. 2001; Armstrong et al. 2003; Miller 2007; Watson et al. 2007), was used to assess the disease risk for a specific geographic area and for a specific species. A list of known diseases was created and, according to the available information, diseases considered most likely to contribute to patterns of population decline observed in woylies in south-west Western Australia were prioritised. One of the main strengths of this process has been the multidisciplinary collaborative approach that was used. In fact, the involvement of scientists from different areas of expertise overcame

the risk that a personal point of view and/or experience would become the main reasoning of the assessment.

The outcome of this collaborative process is that additional diseases to those that have been already tested during the first phase of the WCRP have been highlighted on the basis of the information reported in the literature and recent outcomes of the fieldwork, for further investigation.

Future research may reveal whether there is any association between the seroprevalence of the selected viruses in the declining populations compared to apparently unaffected populations. However, it will still be necessary to detect the pathogens in affected animals and experimental trials will be required before a role for the virus can be clearly established (Caughley 1994; Tompkins et al. 2002). Nevertheless, it is believed that this investigation will generate valuable information with regards to the woylie decline and also provide baseline data for future monitoring and management activities for the woylie and other sympatric species. Additionally, data generated through the suggested investigations will also provide valuable information to build exploratory epidemiological models. The use of population viability analysis (PVA) integrating results of ongoing disease investigations has been suggested in the literature (Haydon et al. 2002; Miller 2007) and it is anticipated that this tool could provide useful information to assist in the identification of the cause(s) of woylie decline, as well as support management decisions. Once it is determined that sufficient data are available on woylies to create reliable exploratory models, it will be possible to take a further step and develop a quantitative risk assessment (Armstrong et al. 2003; Miller 2007). However, a thorough understanding of the species' population dynamics, as well as epidemiological parameters is needed in order to successfully complete this task (Miller 2007).

Lastly, additional information on the general health and its modification in relation to the recent decline in woylie populations would contribute to the decision process.

Table 2.1 List of selected diseases considered in the disease risk assessment in the woylie: bacteria

		Known Presence							Pathogenic Potential			
Disease/Pathogen	Species reported	Captive Populations	Wild Populations	Transmission	Clinical signs	Lesions	Comments	Reference	Likely Presence	Independent	In concert	Risk/Priority
Lumpy jaw	Macropodoidea	Yes	*				Clinical signs would be evident. Unlikely to be responsible for a rapid population decline. Body condition should be poor.	(Speare et al. 1989)	moderate	low	low	low
Mycobacterium spp.	Macropodoidea	Yes, including Woylie	Unknown		Abscesses, skin ulcers, dyspnea, neurological signs		Never noted in free-ranging. Chronic disease.	(Speare et al. 1989)	moderate	moderate	moderate	low
Tetanus	Macropodoidea	*	*	Wounds	Convulsions, muscle stiffness, death		Uncommon in wildlife. More common in northern Australia.	(Speare et al. 1989; Holz 2003)	low	high	nil	low
Salmonella spp.	Macropodoidea	Yes	Yes, but no clinical cases				Commonly isolated from macropods.	(Speare et al. 1989)	moderate/ high	moderate	moderate/ high	moderate/ high
Leptospirosis	Macropodoidea	Yes	Yes	Rodents	Uncommon	Focal interstitial nephritis	Uncommon, no clinical disease in wildlife. No identified in Western Australia.	(Speare et al. 1989)	very low	low	low	low
Listeriosis	Macropodoidea	Yes	Unknown			Hepatic focal Abscesses		(Canfield & Hartley 1992)	low	moderate	moderate/ high	low
Clostridium piliforme (Tyzzer's disease)	Possums, dasyurids, Wombat, koala	Yes	Brushtail and ringtail possum		Sudden death or diarrhoea/anorexia	Necrotizing hepatitis and myocarditis	Wild animals from Sydney. Potentially persistent in the soil.	(Holz 2003)	low	moderate	moderate/ high	low
Pseudomonas pseudomallei (Burkholderia pseudomallei)	Tree-kangaroos	*	Yes		Melioidosis, nasal discharge	Multifocal abscesses	More common in northern Australia.	(Jakob-Hoff 1993; Holz 2003)	low	low	low	low
Pseudomonas pyocyanea	Possums, macropods, koala	*	*			Liquid in the pouch	Pouch is regularly checked.	(Holz 2003)	low	low	low	low
Pseudomonas spp.	Macropodoidea	Joeys	*		Pneumonias		Identified only in captive animals.	(Jackson 2003)	low	moderate	moderate/ high	low
Escherichia coli	Macropodoidea	Joeys	Unknown		Pneumonias		Identified only in captive animals.	(Jackson 2003)	low	moderate	moderate/ high	low
Klebsiella spp.	Macropodoidea	Joeys	*		Pneumonias		Identified only in captive animals.	(Jackson 2003)	low	moderate	moderate/ high	low
Yersinia pseudotuberculosis	Possums	*	*	Rodents and birds	Diarrhoea	Enteritis, septicaemia, hepatic , splenic and renal abscesses		(Holz 2003)	low	moderate/ high	moderate/ high	low
Coxiella burnetti	Macropodoidea	*	Yes	Ticks			Sub-clinical infection in free-ranging macropods in Queensland and Tasmania	(Speare et al. 1989)	very low	low	low	very low
Rickettsia rickettsii	Macropodoidea	*	Yes	Ticks			Queensland	(Speare et al. 1989)	low	moderate/ high	high	low
Chlamydiales	A variety of marsupials	*	Yes		Conjunctivitis, rhinitis, pneumonia, pelvic inflammatory disease, infertility.		Isolated from wild animals from Dryandra (western barred bandicoots, bilbies) Albany (Gilbert's potoroos) and Victoria (mountain brushtail possums, *Trichosurus caninus*). Many species carried without clinical symptoms.	(Bodetti et al. 2003)	moderate	moderate	low/ moderate	moderate

*Not clearly reported in the literature. ^Insufficient data available.

Table 2.2 List of selected diseases considered in the disease risk assessment in the woylie: virus

Disease/Pathogen	Species reported	Known Presence		Transmission	Clinical signs	Lesions	Comments	Reference	Pathogenic Potential			
		Captive Populations	Wild Populations						Likely Presence	Independent	In concert	Risk/Priority
Macropod Herpesvirus	Macropodoidea; wombats	Yes	Yes, serologically positive		Conjunctivitis, dyspnoea, vesicles and ulceration oral cavity, cloacae and genital tract, death. In wallabies-cases of reduce reproduction success (Finnie 1980)	Multifocal necrosis, intranuclear inclusion bodies in various organs. Liver lesions in Woylie (Canfield & Hartley 1992)	No clinical disease reported in wild animals, but presence of sero-positive wild animals. Clinical sings should be evident.	(Dickson et al. 1980; Speare et al. 1989; Holz 2003)	high	moderate	moderate/ high	moderate/ high
Macropod Pox Virus	Macropodoidea	*	Yes	Skin, mosquitoes	Resemble papillomas on tail, dorsum, lip and legs	Eosinophilic cytoplasmic inclusion bodies in epithelial cells		(Speare et al. 1989; Holz 2003)	low	low	low	low
Arboviruvs	Macropodoidea	*	Yes, serologically positive				Viraemia but not clinically ill.	(Speare et al. 1989)	moderate/ high	low	low	low
Ross River Virus	Macropodoidea	Yes	Yes, serologically positive	Mosquitoes	Fever, rash, polyarthritis		Human health risk. Macropods seem to be reservoirs.	(Old & Deane 2005)	moderate/ high	low	low	low
Encephalomyocarditis Virus	Tree kangaroo; quokka	Yes, Tree kangaroo	Ingestion	Rodents	Sudden death, dyspnoea	Pulmonary congestion and oedema, myocarditis		(Quinn et al. 2002; Holz 2003; Jackson 2003)	moderate/ high	moderate	moderate	moderate/ high
Orbivirus (Wallal and Warrego serogroup)	Macropodoidea	*	Yes	*Culicoides* spp.	Chorioretinitis, blindness, circling	Nonsupperative panuveitis, retinitis	Epidemics in Western Australia (Esperance, Albany, Perth) in 94-96 (Hooper 1999).	(Holz 2003; Jackson 2003)	moderate/ high	moderate	moderate/ high	moderate/ high
Orbivirus (Eubenangee serogroup)	Tammar wallaby	*	*	*Culicoides* spp.	Death, muscles fasciculations	Oedema hind limbs, haemorrhage adductors, thorax, cervical area and retroperitoneally. Necrosis of lymphoid germinal centres, gastric ulceration and periacinar hepatic necrosis		(Holz 2003)	moderate/ high	moderate	moderate/ high	moderate/ high
Adenovirus	Macropodoidea	*	Yes, in *B. gaimardi*		None	Enlarge epithelial cells with intranuclear inclusion bodies		(Speare et al. 1989; Holz 2003)	low	moderate	moderate	low
Wobbly possum syndrome, envelope RNA Virus	Brush-tail possum in NZ	*	*	Blood, faeces, urine and mites				(Holz 2003)	very low	moderate	very low	very low

*Not clearly reported in the literature. ^Insufficient data available.

Table 2.3 List of selected diseases considered in the disease risk assessment in the woylie: fungus, protozoa and toxicosis.

		Known Presence							Pathogenic Potential			
Disease/Pathogen	Species reported	Captive Populations	Wild Populations	Transmission	Clinical signs	Lesions	Comments	Reference	Likely Presence	Independent	In concert	Risk/Priority
Fungus												
Candida	Macropodoidea	Yes	*	Contact			Not reported in wild animals.	(Jackson 2003)	high	low	low	low
Dermatophytosis	Macropodoidea	Yes	Unknown	Contact			Not reported in wild animals.	(Speare et al. 1989)	high	low	low/ moderate	low
Cryptococcosis	Potoroos	Yes	Yes	Inhalation	Respiratory signs and meningitis			(Vaughan et al. 2005)	moderate	moderate	moderate	low
Protozoa												
Neospora caninum		Unknown	Unknown		Neurological signs in dogs. Abortion in cattle		Not reported in wild animals.	(Reichel 2000)	moderate/ high	^	^	low
Leishmaniasis	Red kangaroos	Yes	Unknown	Unknown in Australia			Identified near Darwin.	(Rose et al. 2004; Spratt 2005b)	low	low	moderate	low
Toxicosis												
Fluoroacetate (compound 1080)	All Western Australia fauna tolerate high concentration	Yes	Yes			No specific lesions detected at autopsy	Macropods (particularly pouch young) are susceptible. Unlikely: woylie are resistant up to 100 mg/kg.	(King et al. 1981; Speare et al. 1989)	high	very low	low	very low
Phalaris staggers	Macropodoidea	*	*				Isolated cases.	(Speare et al. 1989)	low	low	very low	low
Pyrrolizidine alkaloids	Macropodoidea	*	*				Isolated cases.	(Speare et al. 1989)	low	low	very low	low
Sodium deficiency	Macropodoidea	*	*				Related to low concentration in soil and vegetation.	(Speare et al. 1989)	very low	very low	very low	very low

*Not clearly reported in the literature. ^Insufficient data available.

3 General health of woylie populations in Western Australia

3.1 Introduction

Health assessment and disease investigations of animals at the individual and population level are dependent on the ability to accurately detect variation of the health status. However, with wildlife it can be particularly challenging to determine the health condition of individuals, since it may be difficult to establish reference ranges for physiological parameters and define what is considered to be "normal" (Wobeser 2007). Whilst emerging infectious diseases that are associated with high mortality rates in wildlife populations are considered to be of great concern, the importance of diseases that result in chronic low level mortality or that have sub-lethal effects on the population, for example by reducing fertility or overall survival, should not be underestimated (Spalding & Forrester 1993). Reduction of fitness by pathogens which only result in mild clinical symptoms or which can be potentially carried by individuals subclinically, is in fact, very hard to detect. Additionally, it is often very difficult to sample wild animals during different stages of a pathological process because survival of such animals at advanced stages of disease is reduced and the samples collected may be biased towards healthier animals. Consequently, relating what could seem an unremarkable finding to a preliminary stage of a serious clinical condition may be a challenge.

In several wildlife studies, haematological changes, which were the only detectable abnormality in the study, were associated with reduced survival rates, despite the inability to

clearly identify the underlining pathological process (e.g. Mathews et al. 2006). In fact, haematology is believed to be a reliable indicator of general health in many mammals (Coles 1986; Kerr 2002; Clark 2004). This consideration holds true also for macropod species, despite the fact that there has only been limited research on haematological responses in these species, and that occasionally, animals with severe clinical signs might not show haematological changes of the magnitude expected in other eutherian mammals (Vogelnest & Portas 2008).

Since there was indirect evidence that a disease may have been involved in the woylie decline (Wayne 2006. See also Chapter 1), haematological data and information recorded during the physical examination were analysed to determine whether changes in the general health of woylies were associated with the decline.

The specific aims of this study were to:

1. Establish haematological reference ranges;
2. Investigate differences in haematological parameters and association with the results of physical examinations in woylies from different locations (Upper Warren and Karakamia), populations within Upper Warren (i.e. Kingston and Perup as established by the genetics analysis, Chapter 6, Figure 6.2), gender and seasons.
3. Establish if there were associations between changes in the general health of woylies and demographic dynamics.
4. Assess whether particular factors (geographic or demographic) were associated with an increased risk of having haematological alterations.

3.2 Methods

A total of 966 woylies were trapped using standard wire traps and universal bait (DEC Science Division 2008b) and identified by ear tags, between March 2006 and June 2009, in Karakamia Sanctuary and nine forest blocks in Upper Warren (Table 3.1). Details of the trapping design and frequencies of trapping sessions are reported in the WCRP field operation handbook (DEC Science Division 2008b). Woylies were handled slightly differently between the two locations. In Upper Warren animals were processed immediately after clearing the traps early in the morning, whereas in Karakamia, traps were checked multiple times during the night, woylies bagged and held overnight in bags at a field station, with processing undertaken in the morning.

Weight, head length, gender, reproductive status (including presence of pouch young) and age (classified as juvenile, subadult and adult) were recorded according to the WCRP field operation handbook (DEC Science Division 2008b). Additionally, a physical examination was carried out in the field (DEC Science Division 2008b). However, only evaluations of eye (including the peri-ocular area), ear and rump regions were regularly reported in the clinical health forms, and consequently only these variables were used for the analysis after being recoded as a binomial variable: “normal” for animals that did not have any abnormalities reported in any of these three areas or “abnormal” for any kind of abnormality in at least one of the three body regions examined.

Demographic trends were also considered in some analyses. Dr A. Wayne (DEC) provided long term demographic data for Upper Warren populations. Trapping success (TS: the proportion of woylies trapped divided by the total number of traps available in a trapping session) was used as an index of population abundance, as it was shown that this index correlated well with

woylie abundance (Wayne 2006). Additionally, four different estimates of population decline were used:

- **Annualised absolute difference in TS:** absolute difference in TS from the previous trapping session per year (current TS-previous TS) X (365/time interval in days).
- **Annualised percentage difference in TS:** Percentage change in TS from the previous trapping session per year [1- (previous TS/current TS)] X (365/time interval in days).
- **Absolute difference in TS from peak:** absolute difference in TS from peak (current TS - peak TS), where peak TS is defined as the highest TS recorded for that block.
- **Percentage difference in TS from peak:** Percentage change in TS from peak [1-(current TS/peak TS)].

The first two estimates express the absolute (the actual decline in TS value) and relative (percentage of decline in TS value) "rate" of decline standardised by the time between trapping intervals. The last two estimates indicate the absolute extent and relative proportion of the overall decline.

Table 3.1 Number of captured woylies in each forest block within Upper Warren and Karakamia.

	Upper Warren Blocks											
	BBN	BCP	CBL	CHP	CMR	KNP	MPN	WJP	WRP	Perup	Kingston	Karakamia
Males	76	12	20	9	2	218	6	15	101	323	136	108
Females	26	8	15	7	0	131	4	6	74	176	95	125
Total	102	20	35	16	2	349	10	21	175	499	231	233
Unknown	0	0	0	0	0	2	0	0	1	2	1	0
Grand Total	102	20	35	16	2	351	10	21	176	501	232	233

BBN = Balban; BCP = Boyicup; CBL = Corbal; CHP = Chariup; CMR = Camelar; KNP = Keninup; MPN = Moopinup; WJP = Winnejup; WRP = Warrup; see Figure 1.3 for geographical location of forest blocks.

Blood samples were collected from the lateral tail vein of all woylies that were in poor condition and a subset of apparently healthy individuals. Blood was mixed with EDTA in

commercial tubes and kept on ice in the field (Table 3.2). Two or three blood smears were made at the time of collection and air dried. Upon return from the field, blood samples were submitted to Murdoch University Clinical Pathology Laboratory (on wet ice) and processed within 36 hours of collection, as recommended for macropod blood samples (Hulme-Moir et al. 2006). Differential white blood cell counts were carried out manually by examination of blood smears using light microscopy by the Murdoch University's Clinical Pathology Department, while other parameters were established with an automatic haematology analyser (ADVIA-120) using a multispecies software (Bayer diagnostics division, Tarrytown, NY,USA) including: total white blood cells (WBC, 10^9/L) and red blood cells (RBC, 10^{12}/L) count, haemoglobin concentration (HGB, g/L), mean cell volume (MCV, f/L), mean corpuscular haemoglobin (MCH, p/g), mean corpuscular haemoglobin concentration (MCHC, g/L), corpuscular haemoglobin concentration mean (CHCM: a MCHC direct measurement determined by flowcytometric signal, Bosch et al. 1992), haematocrit (HCT, calculated from RBC count and MCV), red cell distribution width (RDW, %), haemoglobin distribution width (HDW, %), platelet count and mean platelet volume (MPV). However, aggregates of platelets were seen frequently on blood smears and consequently platelet counts and MPV were not considered in further analyses. Total protein (TP, g/L) concentration was assessed by refractometry and fibrinogen (FBG, g/L) concentration by heat precipitation (Coles 1986). Packed cell volume (PCV, %) was also determined after centrifugation of a capillary tube. The ratio of neutrophils to lymphocytes (N:L) has been used in some studies as an indicator of health in marsupial species (e.g. Presidente & Correa 1981). However, because of the considerable variability of these parameters within individuals and inconsistent results, its use is discouraged, especially in macropods (McKenzie et al. 2002; Clark 2004; Young & Deane 2006), therefore N:L was not calculated and it will not be discussed. The HCT and PCV, as well as MCHC and CHCM, were plotted and graphically checked for consistency (Bosch et al. 1992) and, when discordant, blood samples were removed from the dataset together with samples

that showed macroscopic signs of haemolysis (e.g. pink or red plasma). Eventually, during the statistical analysis, only PCV and CHCM were used.

Table 3.2 The number of blood samples collected from each population.

	Kingston	Perup	Karakamia	Total
Males	47	130	17	194
Females	38	77	28	143
Total	85	207	45	337

Normal haematological reference ranges were established between the 5^{th} and 95^{th} percentile of each parameter distribution after removing animals that were considered to be unwell and outliers for the adult age class (Lumsden & Mullen 1978). Generally, 2.5^{th} and 97.5^{th} percentiles are used elsewhere to establish reference ranges (Lumsden & Mullen 1978); however, a more conservative approach was considered to be appropriate due to the relatively small sample size.

Several criteria were considered before an animal was judged to be suitable for the calculation of the haematological reference ranges. It had to have valid morphological measurements and be considered in "good" body condition (see below). Dr N. Parameswaran kindly made available data on the serological status of 355 woylies against *Toxoplasma* (Parameswaran 2008) and only samples from animals with negative results were included for the reference range calculations. Only for Upper Warren, woylies needed to be re-trapped in following trapping sessions (no trapping history was available for woylies in Karakamia because animals trapped were sourced for a translocation).

To establish the animal condition, a biometric index was calculated as the ratio of the weight to the head length. The minimum threshold to consider a woylie in "good" condition was established by calculating the 5^{th} percentile after controlling for age and removing outliers. When there was a statistical difference between locations or genders, these groups were considered separately.

The established haematological reference ranges were then used to assist with the identification of sick woylies. Individuals that showed altered blood values were categorised as:

Anaemic: any animals that had low RBC concentration, HGB and PCV;

Inflammation: any animals that showed changes consistent with an inflammatory response, including increased WBC, neutrophil or monocyte count;

Eosinophilic: any animals that had an increased eosinophil count, with or without increased WBC;

Lymphocytotic: any animals that showed an increased lymphocyte count, with or without an increased WBC.

Due to the unavoidable subjective nature of this classification, a subset of animals (62 individuals) was cross checked by an experienced wildlife clinician (Dr Paul Eden, Perth Zoo). Only two samples (3.2%) were classified discordantly. In both cases, the animals' values were only slightly different from the reference ranges for one blood parameter and whilst the dataset created for this study had not included these animals in any category, the external reviewer had included them in the abnormal category. Given the overall consistent agreement in the clinical judgement, the established dataset was considered reliable and suitable for further analysis. The limited discrepancies indicated that the present dataset represented a conservative approach, reducing, on one hand, the resolution of this health investigation, but,

on the other hand, providing more weight (reducing type II error) when significant results were detected.

3.2.1 Statistical analysis

All the statistical analyses carried out in this study assumed samples were independent to each other, consequently, when multiple entries for the same individual were present (i.e. the same individual was re-sampled), one of them was randomly selected for inclusion in the analysis. The distributions of the variables (all blood parameters and biometric indices) were plotted and visually inspected and the Kolmagorov-Smirnof test of normality was used to confirm a normal distribution. If the variables were normally distributed, the Student's t-test or one way ANOVA was used to compare continuous variables in two or more groups respectively, after establishing whether the assumption of homogeneity of variance was met with the Levene's test. In cases where the assumption of homogeneity of variance was violated, an adjusted t-test value or Welch and Brown-Forsythe robust tests of equality of means were considered, as suggested by Pallant (2007). Post hoc analysis was carried out with the Tukey's HSD test with a set statistical significance of 0.05. For variables that did not have a normal distribution, the non-parametric Mann-Whitney U-test or Kruskal-Wallis test were used to compare two or more groups respectively. When multiple Mann-Whitney U-tests were used, Benjamini-Hochberg approach (Benjamini & Hochberg 1995) was used to control for type I error (see "*Multiple comparisons and type I error*"). Comparisons between location (Upper Warren and Karakamia), populations (Kingston and Perup), gender and seasons were carried out removing animals that were judged to be unwell and outliers. As a result of the limited sample size, comparisons of the same season between different years were not attempted and data from the four years of the study were pooled together.

In order to assess the relative contribution of different predictors, a standard multiple regression was used. Preliminary analyses were carried out to verify compliance with the modelling assumptions (absence of multicollinearity and singularity of variables; normality, linearity, homoscedasticity and independence of residuals). Also, presence of outliers and their influence on the result of the model was controlled by inspection of the scatter plot of the standardised residuals, Mahalanobis and Cook's distances following Tabachnick and Fidell's (2007) guidelines. Because variable distributions tend to normality when the samples size is greater than 30 (Pallant 2007; Field 2009), it was considered appropriate to use multiple regression analysis also for non-normally distributed variables (i.e. PCV and Eosinophil count, n=228) after ensuring that the plot of the regression standardised residual and the scatter plot of the standardised residuals did not depart from normality, linearity and homoscedasticity of residuals.

Differences between prevalences were estimated using the chi-square test for independence and odds ratios were calculated as quantification of the risks along with the 95% or 98.3% confidence interval, depending on whether one or three comparisons were carried out within each risk analysis (see "*Multiple comparisons and type I error*"). If some of the cells in the contingency table reported a count less than five, then the Fisher's exact test was used to identify whether there was a statistical difference in the proportion of the two conditions (Thrusfield 2007).

The possible association between specific blood parameters (RBC, HGB, PCV, TP, WBC, neutrophils, lymphocytes, monocytes and eosinophils) and population abundance, rates of decline (annualised absolute difference in TS and annualised percentage difference in TS) and the overall extent of the decline (absolute difference in TS from peak and percentage

difference in TS from peak) were investigated by calculating Spearman's rho correlation coefficients (r_s).

Additionally, in order to verify the hypothesis that not only the prevalence of apparent health problems, but also the extent of these problems increased over time, the category "health problem" was recoded into an ordinal variable from zero to three, depending on the number of body regions (eyes, ears and rump) that were involved and then tested comparing the group "zero" (no abnormalities) versus the group "three" (presence of health problems that involved eyes, ears and rump) over time (years).

All statistical analyses were carried out in SPSS statistics ver 17 (SPSS Inc.).

3.2.2 Multiple comparisons and type I error

It is well recognised that conducting a large number of statistical analyses increases the probability of type I errors (rejecting the null hypothesis when it is true), i.e. by chance alone the p-values may be lower than the *a priori* established threshold, usually α=0.05 (Miller 1981; Benjamini & Hochberg 1995). An adjustment of α, to establish whether the value found is truly significant or not, has been recommended, with the trade-off of a reduced statistical power. Various approaches have been attempted in order to reduce the lack of power after controlling for type I error (e.g. Simes 1986; Tarone 1990; Benjamini & Hochberg 1995). However, in medical statistics this approach has been heavily criticised (e.g. Rothman 1990; Perneger 1998) given the number of laboratory tests that are generally involved to evaluate the health of individuals. The total number of comparisons is inevitably high and, if a systematic adjustment is applied to reduce type I error, it will, in contrast, increase the

probability of type II error (accepting the null hypothesis when it is false) to an unacceptable level (Rothman 1990; Perneger 1998). Additionally, it has been claimed that the increase of type I error is of concern only with the "universal null hypothesis" that the two groups tested differ concurrently for all the variables considered (Rothman 1990; Perneger 1998). In medical research, instead, each variable is interpreted as different aspects of the health of an individual and a clinical evaluation of the results has been suggested as the most correct control of type I error (Perneger 1998). For example, if anaemia is suspected to occur, a simultaneous decrease of RBC, HGB and PCV will be expected in the presence of normal TP. If these three variables have significant p-values but they go in opposite directions (e.g. one decreases and the other two increase), then there is no clinical relevance in the results despite the statistical significance.

Nevertheless, a correction of α when there are statistical tests that involve the same variables in different groups (i.e. more than two) is still recommended, particularly when a large number of tests are involved, as was the case in this study. Control of type I error was achieved with the Benjamini-Hochberg approach (Benjamini & Hochberg 1995). The new threshold of significance was established as iq/m where i is the i-th observed p-value (sorted from the smallest to the largest), q is the proportion of false significant tests (in this study 0.05) and *m is* the total number of tests carried out for a logically discrete group of analyses (see Appendix 1 for a summary). It is important to note that when the total number of tests is two, the adjustment carried out to protect against type I error with the Benjamini-Hochberg approach (Benjamini & Hochberg 1995) is identical to the sequential Bonferroni correction (Holm 1979). As the number of tests increase, the Benjamini-Hochberg approach (Benjamini & Hochberg 1995) controls the rate of "false discovery" (i.e. the rate of wrongly rejected null hypothesis) while limiting the reduction of statistical power.

3.3 Results

3.3.1 Biometrics

Biometric indices were normally distributed within each age class. Only one juvenile was trapped in Karakamia, consequently, the 5th percentile of the biometric index distribution in this age class was reported only for Upper Warren animals (Table 3.3). No difference was evident between males and females ($p>0.05$) in the juvenile age class. Interestingly, there was a significant difference between males and females in the subadult ($p=0.001$) and adult ($p<0.0005$) age classes only within Upper Warren. Subadult males from Upper Warren had a significantly higher mean biometric index than Karakamia ($p<0.0005$), while adults of both genders each had a significantly higher mean biometric index at Upper Warren relative to Karakamia (males: $p<0.0005$; females: $p<0.0005$). Woylies in Kingston had a moderate but significantly higher mean biometric index than woylies in Perup (males: Kingston, n=117, mean 16.59, SD 1.43; Perup, n=277, mean=16.14, SD=1.29, $p=0.002$. Females: Kingston, n=85, mean 17.12, SD 1.55; Perup, n=152, mean=16.55, SD=1.88, $p=0.018$). There was no difference between females with and without pouch young or between seasons (autumn: March-May; winter: June-August; spring: September-November; summer: December-February).

Table 3.3 Percentiles for biometric condition indeces for males and females after removal of outliers.

	Karakamia				Upper Warren			
Age	n	5th percentile	n	5th percentile	n	5th percentile	N	5th percentile
Juvenile					55#	2.804		
		Males		**Females**		**Males**		**Females**
Subadults	6	7.424	9*	8.190	34	9.854	9*	8.190
Adults	86^	11.136			394	14.155	237	13.899

* Females from both locations were pooled together since there was no significant difference between locations; ^ Males and females were pooled together because there was no statistical difference between genders; # No juveniles were trapped in Karakamia. Since there was no difference between gender, males and females were pooled together

3.3.2 Reference ranges and variation of haematological parameters

3.3.2.1 Location and gender

All haematological parameters were normally distributed except for neutrophils, band neutrophils, monocytes, eosinophils, basophils, HDW and PCV within each location. Only CHCM was significantly different between males and females in Karakamia ($p=0.032$) while RBC concentration ($p=0.028$), RDW ($p=0.006$), WBC ($p=0.029$) and lymphocyte concentrations ($p=0.048$) were different between the two genders in Upper Warren (Tables 3.4-3.5).

Some differences were evident between locations. Within the erythron panel, males in Upper Warren had higher RDW ($p=0.003$) and both genders had lower HDW (males: $p=0.033$; females: $p<0.0005$) when compared with males and females in Karakamia respectively. Additionally, females in Upper Warren had a higher CHCM ($p<0.0005$). The TP concentration was lower in Upper Warren in both genders (males: $p=0.003$; females: $p<0.0005$). In the

leukocyte panel, both genders in Upper Warren had higher WBC (males: $p=0.002$; females: $p<0.0005$), lymphocyte (males: $p<0.0005$; females: $p<0.0005$) and eosinophil (males: $p<0.0005$; females: $p<0.0005$) counts. Additionally, males in Upper Warren also had a higher monocyte concentration ($p= 0.01$) than males in Karakamia.

Due to these statistical differences, haematological reference ranges were calculated separately for each location (Upper Warren and Karakamia). Moreover, because of the differences between males and females in Upper Warren, the reference ranges for the genders were derived separately. Further genetically evident substructure within Upper Warren was not considered when establishing haematological reference ranges (although differences between the two populations were investigated). Woylies disperse regularly between the two compartments of the forest in Upper Warren (Chapter 6) and clinicians may not know the population of origin of the individual being assessed. The total number of individuals considered for the calculation of the reference ranges, after the application of the selection criteria, descriptive statistics and reference ranges (as 5^{th} and 95^{th} percentiles) are summarised in Tables 3.4 and 3.5.

Table 3.4 Median, mean, standard deviation (SD) and 5th and 95th percentiles of haematological parameters in the Upper Warren populations.

	Males					Females				
					Percentiles					Percentiles
Parameter	N	Median	Mean	SD	5	95	N	Median	Mean	SD	5	95
WBC (10^9/L)	69	5.30	5.53	2.01	2.55	9.40	41	4.70	4.72	1.51	2.61	7.58
RBC (10^{12}/L)	69	11.49	11.37	1.16	9.62	13.50	41	10.65	10.86	1.19	8.85	12.80
HGB (g/L)	69	161.00	158.78	14.45	129.50	179.50	41	157.00	155.24	11.80	132.60	170.90
MCV (f/L)	69	45.30	46.12	3.52	41.40	51.90	41	46.40	46.88	4.02	40.16	53.30
MCH (p/g)	69	13.90	14.02	1.20	12.30	16.20	41	14.20	14.39	1.25	12.41	17.40
CHCM (g/L)	69	301.00	301.64	13.74	276.50	324.00	41	307.00	305.76	13.70	283.20	329.60
RDW (%)	69	11.60	11.57	0.75	10.15	12.85	41	11.10	11.18	0.60	10.11	12.28
HDW (%)	69	15.60	15.77	1.28	13.65	18.15	41	15.70	15.92	1.49	13.54	18.46
Neutrophil(10^9/L)	69	1.71	1.96	1.07	0.66	4.31	41	1.76	1.80	0.79	0.68	3.18
Band Neutrophil	69	0.00	0.00	0.02	0.00	0.05	41	0.00	0.00	0.01	0.00	0.06
Lymphocyte(10^9/L)	69	2.98	3.03	1.59	0.65	5.76	41	2.58	2.45	1.28	0.58	5.00
Monocyte (10^9/L)	69	0.10	0.12	0.08	0.00	0.29	41	0.08	0.12	0.11	0.00	0.39
Eosinophil (10^9/L)	69	0.13	0.21	0.21	0.00	0.67	41	0.09	0.15	0.16	0.00	0.40
Basophil (10^9/L)	69	0.00	0.03	0.05	0.00	0.13	41	0.00	0.02	0.03	0.00	0.09
PCV (%)	64	0.49	0.48	0.04	0.40	0.55	40	0.48	0.47	0.04	0.40	0.52
TP (g/L)	64	66.00	66.19	4.72	58.00	74.00	40	65.50	66.03	3.78	60.00	71.95
FBG (g/L)	12	2.00	2.25	0.62			9	2.00	2.22	0.67		

N: number of animals screened

Table 3.5 Median, mean, standard deviation (SD) and 5th and 95th percentiles of haematological parameters in Karakamia.

					Percentiles	
Parameter	N	Median	Mean	SD	5	95
WBC (10^9/L)	34	3.25	3.24	1.20	1.38	5.63
RBC (10^{12}/L)	34	11.09	11.14	0.83	9.63	12.63
HGB (g/L)	34	165.00	164.76	7.88	150.50	177.75
MCV (f/L)	34	47.80	47.55	2.35	44.13	51.80
MCH (p/g)	34	14.65	14.87	1.15	13.45	17.33
CHCM (g/L)	34	296.00	295.12	10.08	276.75	314.00
RDW (%)	34	11.10	11.02	0.57	10.05	11.83
HDW (%)	34	19.25	18.53	2.46	13.83	21.43
Neutrophil(10^9/L)	28	1.61	1.92	1.22	0.54	4.68
Band Neutrophil	28	0.00	0.08	0.41	0.00	1.20
Lymphocyte(10^9/L)	28	1.08	1.16	0.58	0.29	2.65
Monocyte (10^9/L)	28	0.04	0.06	0.06	0.00	0.18
Eosinophil (10^9/L)	28	0.03	0.03	0.04	0.00	0.14
Basophil (10^9/L)	28	0.00	0.01	0.03	0.00	0.12
PCV (%)	34	0.48	0.48	0.03	0.44	0.55
TP (g/L)	34	75.00	74.26	5.70	63.00	81.00
FBG (g/L)	8	2.00	1.88	0.35		

N: number of animals screened.

Within Upper Warren, the two genetically distinct populations (Chapter 6) did not show any differences in the leukocyte panel while erythrocyte concentration was significantly lower (males: $p<0.0005$; females: $p<0.0005$) and MCV and MCH higher (males: $p<0.0005$; females: $p<0.0005$) in Kingston. Males in Kingston also had higher RDW ($p<0.0005$).

3.3.2.2 Seasons

Several haematological differences were found between seasons and these are summarised in Tables 3.6-3.8. General trends in Upper Warren, for most blood parameters, are broadly

consistent between the two genders within the same population and for the same gender across populations, although these are not always significant. There was an overall decreasing pattern in erythrocyte concentration, HGB and PCV from the colder months to the warmer (Tables 3.6-3.7) with no significant changes in TP.

Total leukocyte counts (WBC) seemed to have much greater fluctuation between seasons, with winter and summer being lower than autumn and spring in all groups and locations (see Table 3.6-3.7). When looking at the differential counts, lymphocytes appeared to be responsible for the overall WBC trend in Upper Warren. In fact, in winter, lymphocyte counts were significantly lower in all groups, except females in Kingston. Eosinophils from males in Perup had a significant similar trend to that described for WBC. Nevertheless, it is interesting to note that eosinophil counts from females of the same population showed a similar pattern although this was not significant (Table 3.6).

Table 3.6 Differences in haematological parameters between seasons in males from Upper Warren.

		Males Kingston						Males Perup					
Parameter	Season	N	Mean	SD	p (ANOVA)	Post-hoc pair	Sig	N	Mean	SD	p (ANOVA)	Post-hoc pair	Sig
WBC	Autumn	20	5.61	2.03	0.009	Aut-Wint	0.030	54	6.14	1.63	<0.0005	Aut-Wint	0.005
(10^9/L)	Winter	5	3.12	1.17		Wint-Spring	0.006	14	4.44	1.60		Wint-Spring	0.006
	Spring	16	6.26	1.79				19	6.39	1.89		Spring-Sum	0.005
	Summer							11	4.24	1.49		Aut-Sum	0.004
RBC	Autumn	20	11.38	1.22	<0.0005	Aut-Spring	<0.0005	54	11.66	1.32	0.156		
(10^{12}/L)	Winter	5	10.60	0.71				14	11.81	1.14			
	Spring	16	9.83	0.86				19	11.66	0.95			
	Summer							11	10.81	1.03			
HGB	Autumn	20	161.00	13.00	0.025 (B-F)			54	160.22	15.29	0.128		
(g/L)	Winter	5	164.60	1.34	0.013 (W)			14	163.50	14.82			
	Spring	16	151.38	16.85				19	159.74	9.37			
	Summer							11	150.09	17.28			
MCV	Autumn	20	47.01	3.17	0.042			54	45.37	3.09	0.216 (W)		
(f/L)	Winter	5	50.58	3.70				14	44.38	1.90			
	Spring	16	49.01	2.92				19	44.74	1.88			
	Summer							11	43.85	2.01			
MCH	Autumn	20	14.25	1.35	0.017	Aut-Spring	0.026	54	13.79	0.94	>0.946		
(p/g)	Winter	5	15.58	1.09				14	13.89	1.20			
	Spring	16	15.42	1.27				19	13.73	0.65			
	Summer							11	13.91	0.95			
CHCM	Autumn	20	304.45	17.59	0.457			54	306.15	13.07	0.008 (W)	Wint-Spring	0.041
(g/L)	Winter	5	313.00	8.69				14	309.79	22.02			
	Spring	16	302.75	15.20				19	297.05	8.02			
	Summer							11	302.64	6.28			
RDW	Autumn	20	12.57	1.043	0.002	Aut-Wint	0.003	54	11.40	0.71	0.001	Aut-Wint	<0.0005
(%)	Winter	5	10.98	0.75		Aut-Spring	0.038	14	10.62	0.45		Wint-Spring	0.007
	Spring	16	11.79	0.72				19	11.36	0.58			
	Summer							11	11.13	0.45			
HDW	Autumn	20	16.24	1.84	**0.001** (K-W test)	Aut-Wint	0.003	54	16.15	1.45	**0.008** (K-W test)		
(%)	Winter	5	17.52	1.88		Aut-Spring	0.002	14	16.85	2.02			
	Spring	16	14.54	1.04				19	14.88	1.33			
	Summer							11	15.95	1.05			

		Males Kingston						Males Perup					
Parameter	Season	N	Mean	SD	p (ANOVA)	Post-hoc pair	Sig	N	Mean	SD	p (ANOVA)	Post-hoc pair	Sig
Neutrophil	Autumn	20	1.82	1.04	0.334			54	2.19	1.08	0.344		
(10^9/L)	Winter	5	1.43	0.75				14	2.54	1.24			
	Spring	16	2.13	1.21				19	2.01	1.05			
	Summer							11	1.67	0.73			
Lymphocyte	Autumn	20	3.00	1.61	0.009	Wint-Spring	0.006	54	3.22	1.32	<0.0005	Aut-Wint	<0.0005
(10^9/L)	Winter	5	1.14	0.88				14	1.61	0.95		Wint-Spring	<0.0005
	Spring	16	3.55	1.32				19	3.82	1.41		Spring-Sum	0.006
	Summer							11	2.19	1.07			
Monocyte	Autumn	20	0.15	0.13	0.963			54	0.15	0.12	0.102		
(10^9/L)	Winter	5	0.12	0.07				14	0.08	0.09			
	Spring	16	0.13	0.12				19	0.10	0.08			
	Summer							11	0.17	0.17			
Eosinophil	Autumn	20	0.23	0.22	0.879			54	0.23	0.24	**<0.0005** (K-W test)	Aut-Sum	0.017
(10^9/L)	Winter	5	0.27	0.25				14	0.14	0.17		Wint-Spring	0.002
	Spring	16	0.24	0.22				19	0.39	0.25		Spring-Sum	<0.0005
	Summer							11	0.09	0.15			
Basophil	Autumn	20	0.03	0.03	0.971			54	0.03	0.04	0.406		
(10^9/L)	Winter	5	0.03	0.02				14	0.02	0.04			
	Spring	16	0.03	0.04				19	0.02	0.04			
	Summer							11	0.01	0.02			
PCV	Autumn	20	0.49	0.04	**0.002** (K-W Test)	Aut-Spring	<0.0005	51	0.49	0.05	0.456		
(%)	Winter	4	0.48	0.02				13	0.47	0.06			
	Spring	16	0.43	0.04				19	0.47	0.03			
	Summer							10	0.45	0.05			
TP	Autumn	20	67.15	5.37	0.039	Aut-Wint	0.049	51	66.90	4.36	0.049	Aut-Sum	0.037
(g/L)	Winter	4	60.75	4.50				13	65.08	7.20			
	Spring	16	64.50	3.92				19	65.68	2.96			
	Summer							9	62.22	5.40			
FBG	Autumn	8	1.63	0.52	0.011			16	2.13	0.50	0.375 (W)		
(g/L)	Winter	0						0					
	Spring	11	2.45	0.69				9	2.56	1.33			
	Summer							0					

B-F, Brown-Forsythe robust test of equality of means; W, Welch robust test of equality of means; K-W test, Kruskal-Wallis test. In bold, significant p-values after Benjamini-Hochberg correction (Benjamini & Hochberg 1995). Aut, Autumn; Wint, Winter; Sum, Summer.

Table 3.7 Differences in haematological parameters between seasons in females from Upper Warren

		Females Kingston						Females Perup					
Parameter	Season	N	Mean	SD	p (ANOVA)	Post-hoc pair	Sig	N	Mean	SD	p (ANOVA)	Post-hoc pair	Sig
WBC	Autumn	18	5.77	1.71	0.019	Aut-Spring	0.039	35	5.76	1.89	0.002	Wint-Spring	0.001
(10^9/L)	Winter	2	3.45	1.06				11	4.24	1.12			
	Spring	9	4.16	1.06				12	7.04	1.49			
	Summer							3	4.73	1.93			
RBC	Autumn	18	10.45	1.25	0.064			35	11.20	1.03	0.354		
(10^{12}/L)	Winter	2	9.97	0.27				11	11.86	1.09			
	Spring	9	9.41	0.65				12	11.43	1.31			
	Summer							3	11.10	0.50			
HGB	Autumn	18	156.83	15.38	0.257			35	158.29	11.81	0.950		
(g/L)	Winter	2	160.50	10.61				11	156.82	13.84			
	Spring	9	147.67	12.27				12	159.50	11.77			
	Summer							3	156.33	10.97			
MCV	Autumn	18	48.58	3.60	0.287			35	45.75	3.52	0.265		
(f/L)	Winter	2	51.30	2.83				11	43.70	2.50			
	Spring	9	50.46	2.79				12	45.00	1.99			
	Summer							3	43.93	4.31			
MCH	Autumn	18	15.04	1.31	0.330			35	14.18	1.07	0.076		
(p/g)	Winter	2	16.05	0.64				11	13.26	0.73			
	Spring	9	15.74	1.43				12	14.03	0.87			
	Summer							3	14.13	1.62			
CHCM	Autumn	18	310.94	14.61	0.086			32	306.06	15.37	0.053		
(g/L)	Winter	2	321.00	7.07				11	312.55	12.83			
	Spring	9	300.33	12.39				12	296.33	10.62			
	Summer							3	306.00	6.56			
RDW	Autumn	18	11.98	1.19	0.034	NS		32	11.44	0.57	0.005	Aut-Wint	0.021
(%)	Winter	2	10.40	0.57				11	10.89	0.46			
	Spring	9	11.02	0.72				12	11.08	0.38			
	Summer							3	10.63	0.81			
HDW	Autumn	18	16.70	1.57	**0.005** (K-W test)	NS		32	16.43	1.67	**0.001** (K-W test)	Wint-Spring	<0.0005
(%)	Winter	2	16.90	0.14				11	17.36	1.37		Aut-Spring	0.002
	Spring	9	14.87	0.68				12	14.68	1.04			
	Summer							3	16.40	0.63			

		Females Kingston						Females Perup					
Parameter	Season	N	Mean	SD	p (ANOVA)	Post-hoc pair	Sig	N	Mean	SD	p (ANOVA)	Post-hoc pair	Sig
Neutrophil	Autumn	18	2.52	1.06	0.017 (K-W test)	Aut-Spring	0.005	35	2.24	1.05	0.992		
(10^9/L)	Winter	2	1.94	0.71				11	2.26	0.95			
	Spring	9	1.39	0.82				12	2.24	1.25			
	Summer							3	2.24	0.14			
Lymphocyte	Autumn	18	2.62	1.24	0.205			35	3.07	1.54	0.001	Aut-Wint	0.023
(10^9/L)	Winter	2	1.15	0.35				11	1.59	1.08		Wint-Spring	0.001
	Spring	9	2.38	0.73				12	4.04	1.50			
	Summer							3	2.12	1.50			
Monocyte	Autumn	18	0.17	0.15	0.179			35	0.14	0.10	0.939		
(10^9/L)	Winter	2	0.10	0.08				11	0.13	0.08			
	Spring	9	0.07	0.08				12	0.15	0.19			
	Summer							3	0.12	0.03			
Eosinophil	Autumn	18	0.14	0.12	0.564			34	0.13	0.16	0.469		
(10^9/L)	Winter	2	0.22	0.09				11	0.11	0.11			
	Spring	9	0.22	0.18				12	0.20	0.19			
	Summer							3	0.15	0.18			
Basophil	Autumn	18	0.02	0.03	0.055			34	0.03	0.04	0.992		
(10^9/L)	Winter	2	0.06	0.04				11	0.03	0.05			
	Spring	9	0.003	0.01				12	0.03	0.05			
	Summer							3	0.02	0.04			
PCV	Autumn	18	0.47	0.05	0.502			29	0.48	0.04	0.594		
(%)	Winter	2	0.47	0.04				11	0.48	0.04			
	Spring	9	0.45	0.04				12	0.47	0.05			
	Summer							3	0.45	0.03			
TP	Autumn	18	65.72	4.84	0.477			32	67.56	6.13	0.907		
(g/L)	Winter	2	63.50	2.12				11	66.00	7.76			
	Spring	9	65.22	4.79				12	67.50	4.42			
	Summer							3	67.33	6.66			
FBG	Autumn	8	2.00	0.54	0.814			16	2.44	1.46	0.660		
(g/L)	Winter	0						0					
	Spring	2	1.50	0.71				7	2.71	1.11			
	Summer							0					

K-W test, Kruskal-Wallis test. In bold, significant p-values after Benjamini-Hochberg correction (Benjamini & Hochberg 1995)

Table 3.8 Differences in haematological parameters between seasons in Karakamia

Parameter	Season	N	Mean	SD	t-test
WBC	Winter	25	2.81	0.86	<0.0005
(10^9/L)	Spring	8	4.48	1.33	
RBC	Winter	25	11.05	0.86	0.181
(10^{12}/L)	Spring	8	11.51	0.70	
HGB	Winter	25	165.64	6.38	0.504
(g/L)	Spring	8	163.50	11.36	
MCV	Winter	25	48.18	2.20	0.011
(f/L)	Spring	8	45.80	2.00	
MCH	Winter	25	15.08	1.23	0.069
(p/g)	Spring	8	14.23	0.67	
CHCM	Winter	7*	294.86	6.28	0.035
(g/L)	Spring	6*	304.67	8.41	
RDW	Winter	25	11.18	0.47	0.002
(%)	Spring	8	10.49	0.57	
HDW	Winter	25	19.74	1.03	<0.0005 (M-W test)
(%)	Spring	8	14.54	1.02	
Neutrophil	Winter	19	1.42	0.70	0.002 (M-W test)
(10^9/L)	Spring	8	3.25	1.26	
Lymphocyte	Winter	19	1.11	0.54	0.873
(10^9/L)	Spring	8	1.08	0.37	
Monocyte	Winter	19	0.06	0.06	0.391
(10^9/L)	Spring	8	0.07	0.05	
Eosinophil	Winter	19	0.03	0.04	0.579
(10^9/L)	Spring	8	0.03	0.02	
Basophil	Winter	19	0.00	0.01	0.071
(10^9/L)	Spring	8	0.04	0.05	
PCV	Winter	25	0.48	0.03	0.949
(%)	Spring	8	0.48	0.03	
TP	Winter	25	76.68	3.59	<0.0005
(g/L)	Spring	8	66.75	4.83	

* only males. M-W test, Mann-Whitney U-test.

In Karakamia there were significant differences in MCV, CHCM and RDW (Table 3.8) between winter and spring. However, these differences were not consistent with what was found in Upper Warren.

On the other hand, overall WBC changes in Karakamia were consistent with Upper Warren although these did not correspond to the lymphocyte concentration changes as was evident in Upper Warren but rather to a significant increase neutrophil count ($p=0.002$).

To investigate the relative contribution of the gender, season and geographic location within Upper Warren, a multiple regression analysis was carried out using RBC, HGB, PCV, TP, WBC, lymphocyte and eosinophil concentrations as dependent variables. The model explained 18.4% of the variance of RBC concentration ($p<0.0005$). The geographical location (Kingston or Perup) significantly explained the greatest amount of the variance (13.99%; $p<0.0005$). Seasonality was responsible for 3.5% ($p=0.002$) and gender for 2% ($p=0.019$).

The HGB concentration, PCV and TP were significantly predicted by the models (HGB and TP: $p=0.032$; PCV: $p=0.001$) with seasons being the only significant explanatory variable (HGB, $p=0.015$; TP, $p=0.018$; PCV, $p<0.0005$) accounting for 2.6%, 6.35% and 2.5% of variance of HGB, PCV and TP respectively.

No significant models were found with WBC and lymphocyte concentration as dependent variables. The model explained only 4.1% of the variance of eosinophil concentration ($p=0.025$) with gender explaining 3.65% of the variance ($p=0.004$).

3.3.3 Haematological abnormalities and disease risks

3.3.3.1 Location and gender

There was no evidence that a higher proportion of animals in Upper Warren had abnormal blood parameters when compared with woylies from Karakamia, except for animals that

showed lymphocytosis (Fisher's exact test, p=0.044). In Upper Warren, 8% (23) of woylies had lymphocyte counts above the 95th percentile, while none of the woylies trapped in Karakamia had abnormal lymphocyte levels.

When considering only woylies trapped within Upper Warren, animals in Kingston were 15.63 times (95% CI =1.84-125) more likely to be anaemic than animals in Perup. On the other hand, animals in Perup had a higher proportion (11.3%, n=23) of lymphocytosis, than Kingston (Fisher's exact test, p<0.0005). There was no increased risk of having abnormal blood parameters associated with gender.

3.3.3.2 Haematological patterns with respect to the decline and population abundance

Since sufficient samples had been collected in Keninup before the decline started in this area (48 samples pre-decline and 82 during the decline), an analysis was undertaken to evaluate whether there were haematological differences before and during the decline in this area. Both genders showed an increased MCH during the decline (males: pre-decline, n=27, mean 13.11, SD 0.72; during decline, n=49, mean=14.01, SD=0.79, p<0.0005; females: pre-decline, n=21, mean 13.49, SD 0.89; during decline, n=33, mean=14.1, SD=1.1, p=0.036) and males only had an increased MCV (pre-decline, n=27, mean 43.55, SD 2.41; during decline, n=49, mean=45.02, SD=2.04, p=0.006). Additionally, both genders had a reduced PCV during the decline although results were significant only for females (pre-decline, n=20, mean 0.485, SD 0.064; during decline, n=31, mean=0.448, SD=0.046, p=0.022). Nevertheless neither gender had a higher risk of having blood parameters outside the reference ranges during the decline. Likewise, no changes in the biometric index were present.

Only marginal correlations were found with some of the measurements of decline and these are summarised in Tables 3.9-3.10. It is interesting to note that, even if only small to moderate correlations were present, males from both populations showed a positive correlation between WBC counts and rates of decline. However, in Perup these correlations appeared to be related to population densities (TS) in contrast to those calculated for animals from Kingston (Tables 3.9-3.10). Further evidence of the differences between the two populations can be seen by the fact that lymphocyte concentrations were moderately associated with population abundance in Perup, while they were not in Kingston.

Table 3.9 Spearman's rho correlation coefficients between selected haematological parameters, population abundance and decline estimates in Kingston.

		Males Kingston					Females Kingston				
		TS	AAD TS	APD TS	AD TS peak	PD TS peak	TS	AAD TS	APD TS	AD TS peak	PD TS peak
AAD TS	r_s	0.903									
	Sig. (1-tailed)	0.000									
	N	35									
APD TS	r_s	0.685	0.858								
	Sig. (1-tailed)	0.000	0.000								
	N	35	35								
AD TS peak	r_s	0.277	0.170	-0.047							
	Sig. (1-tailed)	0.046	0.164	0.394							
	N	38	35	35							
PD TS peak	r_s	0.657	0.481	0.264	0.721						
	Sig. (1-tailed)	0.000	0.002	0.063	0.000						
	N	38	35	35	38						
WBC	r_s	0.029	0.444	0.479	-0.471	-0.134	-0.276	0.342	0.340	0.029	0.157
(10^9/L)	Sig. (1-tailed)	0.423	0.004	0.002	0.001	0.210	0.049	0.055	0.056	0.447	0.232
	N	47	35	35	38	38	37	23	23	24	24
RBC	r_s	-0.304	-0.173	0.142	-0.199	-0.165	-0.331	0.137	0.236	-0.140	-0.011
(10^{12}/L)	Sig. (1-tailed)	0.019	0.160	0.208	0.116	0.161	0.023	0.267	0.139	0.257	0.479
	N	47	35	35	38	38	37	23	23	24	24
HGB	r_s	-0.029	-0.149	-0.085	0.075	0.213	-0.039	0.326	0.344	0.106	0.251
(g/L)	Sig. (1-tailed)	0.424	0.196	0.314	0.327	0.100	0.408	0.064	0.054	0.310	0.119
	N	47	35	35	38	38	37	23	23	24	24

		Males Kingston					Females Kingston				
		TS	AAD TS	APD TS	AD TS peak	PD TS peak	TS	AAD TS	APD TS	AD TS peak	PD TS peak
Neutrophil (10^9/L)	r_s	0.222	0.386	0.481	-0.114	0.064	0.031	0.450	0.483	0.229	0.357
	Sig. (1-tailed)	0.067	0.011	0.002	0.248	0.352	0.428	0.016	0.010	0.141	0.043
	N	47	35	35	38	38	37	23	23	24	24
Lymphocyte (10^9/L)	r_s	-0.032	0.315	0.199	-0.468	-0.159	-0.445	0.054	0.011	-0.366	-0.347
	Sig. (1-tailed)	0.414	0.032	0.126	0.002	0.170	0.003	0.403	0.480	0.039	0.049
	N	47	35	35	38	38	37	23	23	24	24
Monocyte (10^9/L)	r_s	0.032	0.012	0.051	0.054	0.131	-0.110	0.246	0.304	0.026	0.102
	Sig. (1-tailed)	0.417	0.473	0.385	0.373	0.216	0.259	0.129	0.079	0.452	0.318
	N	47	35	35	38	38	37	23	23	24	24
Eosinophil (10^9/L)	r_s	-0.205	-0.220	-0.328	-0.034	-0.078	-0.052	-0.400	-0.425	0.010	0.004
	Sig. (1-tailed)	0.083	0.102	0.027	0.419	0.320	0.381	0.029	0.021	0.481	0.493
	N	47	35	35	38	38	37	23	23	24	24
PCV (%)	r_s	-0.304	-0.392	-0.124	-0.123	-0.134	0.016	0.285	0.345	0.016	0.178
	Sig. (1-tailed)	0.020	0.011	0.243	0.234	0.214	0.464	0.094	0.053	0.470	0.202
	N	46	34	34	37	37	37	23	23	24	24
TP (g/L)	r_s	-0.119	0.135	0.251	-0.117	-0.178	-0.365	-0.214	-0.206	-0.239	-0.289
	Sig. (1-tailed)	0.215	0.224	0.076	0.245	0.145	0.013	0.164	0.173	0.130	0.085
	N	46	34	34	37	37	37	23	23	24	24

r_s, Spearman's rho correlation coefficients; TS, trapping success; AAD TS, annualised absolute difference in TS; APD TS, annualised percentage difference in TS; AD TS peak, absolute difference in TS from peak; PD TS peak, percentage difference in TS from peak. In bold significant p-values after Benjamini-Hochberg correction (Benjamini & Hochberg 1995)

Table 3.10 Spearman's rho correlation coefficients between selected haematological parameters, population abundance and decline estimates in Perup.

		Males Perup					Females Perup				
		TS	AAD TS	APD TS	AD TS peak	PD TS peak	TS	AAD TS	APD TS	AD TS peak	PD TS peak
AAD TS	r_s	0.533									
	Sig. (1-tailed)	0.000									
	N	111									
APD TS	r_s	0.592	0.973								
	Sig. (1-tailed)	0.000	0.000								
	N	111	111								
AD TS peak	r_s	0.613	-0.003	0.056							
	Sig. (1-tailed)	0.000	0.487	0.280							
	N	126	111	111							
PD TS peak	r_s	0.620	-0.011	0.057	0.987						
	Sig. (1-tailed)	0.000	0.454	0.276	0.000						
	N	126	111	111	126						
WBC	r_s	0.268	0.238	0.227	0.116	0.141	0.188	-0.020	0.005	-0.019	-0.001
(10^9/L)	Sig. (1-tailed)	0.001	0.006	0.008	0.097	0.058	0.051	0.433	0.482	0.434	0.497
	N	126	111	111	126	126	77	71	71	77	77
RBC	r_s	-0.130	0.087	0.071	-0.030	-0.022	-0.012	-0.055	-0.066	-0.027	-0.035
(10^{12}/L)	Sig. (1-tailed)	0.073	0.181	0.230	0.368	0.402	0.459	0.324	0.292	0.407	0.382
	N	126	111	111	126	126	77	71	71	77	77
HGB	r_s	-0.137	-0.036	-0.057	-0.033	-0.034	-0.074	-0.202	-0.188	-0.156	-0.160
(g/L)	Sig. (1-tailed)	0.063	0.353	0.275	0.358	0.352	0.261	0.046	0.059	0.088	0.083
	N	126	111	111	126	126	77	71	71	77	77

		Males Perup					Females Perup				
		TS	AAD TS	APD TS	AD TS peak	PD TS peak	TS	AAD TS	APD TS	AD TS peak	PD TS peak
Neutrophil	r_s	0.204	0.167	0.185	0.059	0.060	-0.127	-0.044	-0.002	-0.169	-0.179
(10^9/L)	Sig. (1-tailed)	0.011	0.040	0.026	0.256	0.251	0.135	0.359	0.495	0.071	0.060
	N	126	111	111	126	126	77	71	71	77	77
Lymphocyte	r_s	0.196	0.247	0.215	0.054	0.080	0.257	0.055	0.062	0.012	0.036
(10^9/L)	Sig. (1-tailed)	0.014	0.005	0.012	0.273	0.186	0.012	0.323	0.304	0.458	0.377
	N	126	111	111	126	126	77	71	71	77	77
Monocyte	r_s	0.037	-0.038	-0.038	0.075	0.094	-0.147	-0.098	-0.080	0.051	0.053
(10^9/L)	Sig. (1-tailed)	0.339	0.348	0.348	0.201	0.147	0.100	0.209	0.253	0.328	0.324
	N	126	111	111	126	126	77	71	71	77	77
Eosinophil	r_s	0.172	0.123	0.101	0.013	0.038	0.216	-0.013	0.014	0.004	0.013
(10^9/L)	Sig. (1-tailed)	0.027	0.099	0.146	0.443	0.338	0.030	0.456	0.454	0.487	0.456
	N	126	111	111	126	126	76	71	71	76	76
PCV	r_s	-0.195	-0.057	-0.121	0.045	0.043	-0.117	-0.065	-0.097	-0.015	-0.024
(%)	Sig. (1-tailed)	0.016	0.281	0.108	0.313	0.318	0.166	0.299	0.214	0.451	0.422
	N	121	106	106	121	121	71	69	69	71	71
TP	r_s	-0.114	-0.057	-0.033	-0.023	0.016	-0.187	-0.149	-0.140	-0.177	-0.176
(g/L)	Sig. (1-tailed)	0.108	0.279	0.367	0.400	0.430	0.055	0.111	0.126	0.066	0.067
	N	120	106	106	120	120	74	69	69	74	74

r_s, Spearman's rho correlation coefficients; TS, trapping success; AAD TS, annualised absolute difference in TS; APD TS, annualised percentage difference in TS; AD TS peak, absolute difference in TS from peak; PD TS peak, percentage difference in TS from peak. n bold significant p-values after Benjamini-Hochberg correction (Benjamini & Hochberg 1995)

3.3.3.3 Field health examinations

The majority of health problems reported were various types of skin lesions (Figure 3.1 and 3.2) and, although skin biopsies were collected for histopathological examinations, the histopathology was not part of this research project. Collaborators of the WCRP led the histopathological investigation and preliminary results lacked a clear indication of possible aetiological agents, suggesting that hair loss is, most likely, secondary to irritation and self trauma (P. Nicholls[5], personal communication).

Health problems were present in a higher proportion of woylies from Upper Warren (41.3%, n=557) than those from Karakamia (10%, n=80, $p<0.0005$. OR=6.33, 95% CI 2.99-13.40). No difference was found between the two populations in Upper Warren and, in Perup only, males were more likely to present such lesions (OR= 1.73, 98.3% CI= 1.02-2.94). Age did not influence the risk of having a health problem (OR= 1.8, 95% CI=0.73-4.41) between subadults and adults. Unfortunately, the sample size was too small for juveniles (n=11) to carry out a meaningful analysis for this age class.

[5] Dr Philip Nicholls, Associate Professor, School of Veterinary and Biomedical Sciences (Pathology), Murdoch University.

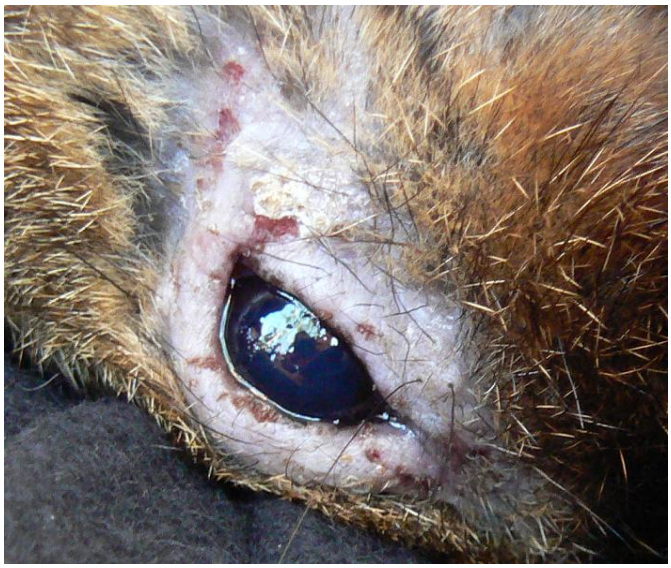

Figure 3.1 Woylie with periocular alopecia and crusting.

(Photo courtesy of Dr A. Wayne)

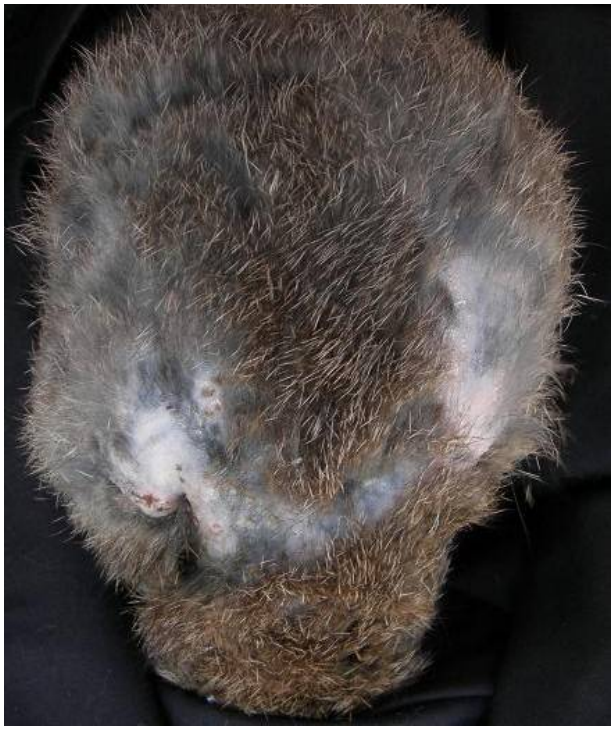

Figure 3.2 Woylie with rump and dorsal tail base alopecia and crusting. Note also the moderate hyperkeratosis of the left side of the dorsal tail base.

(Photo courtesy of Dr A. Wayne)

Although some differences between seasons were present (data not shown), changes between seasons were inconsistent across different years. As an example, in Figure 3.3 the prevalence of health problems in Keninup in different years of the study is reported. Prevalence fluctuations seemed to increase over time and, to test this hypothesis, the prevalence of each year (from autumn to summer of the following calendar year) was compared within the same forest block. Throughout the four year study, the prevalence of health problems increased significantly over time in Keninup ($p<0.0005$) and Balban ($p<0.0005$), while in Warrup, only data collected in the fourth year of the study were statistically different from the previous three ($p<0.0005$, Table 3.11). The number of animals showing concurrent health problems that involved eyes, ears and rump was higher in the last two years of the study (2008 and 2009, $p<0.0005$).

Since the decline progressed throughout this time, it was investigated whether the prevalence of health problems was associated with either the rate of decline or with the absolute decreased woylie abundance using Spearman's rho correlation coefficient. There was a moderate negative correlation between the presence of health problems and the "rate" of decline (Annualised absolute difference in TS and Annualised percentage difference in TS. Table 3.12), while a limited correlation was present between the absolute densities and the overall decline (Table 3.12). In other words, the quicker the population was declining, the higher the prevalence of health problems. Note that rates and the extent of the decline have negative values when the decline is occurring and stabilise around zero when the population reaches a low density.

For a subset of these animals, it was possible to investigate whether the health problems reported were associated with haematological changes. Given the statistical differences that

were evident in the haematological parameters between locations, populations and gender, the analysis was conducted separately for each gender and included only animals trapped within Upper Warren. The results of this analysis revealed only minor differences. In fact, males in Kingston showed a lower MCH (normal, n=23, mean 15.56, SD 1.03; altered, n=11, mean=14.35, SD=1.51, p=0.009), while males in Perup had a higher HDW (normal, n=47, mean 15.33, SD 1.77; altered, n=48, mean=16.28, SD=1.46, p=0.003) and neither gender had a higher risk of having blood parameters outside the reference ranges when animals that had health problems were compared with "normal".

Table 3.11 Distribution of prevalence of health problems in Balban, Keninup and Warrup over the four years of the study (2006-2009).

	Balban		Keninup		Warrup	
Year	%	Total	%	Total	%	Total
2006	33.3	27	17.6	85	37	27
2007	83.3	24	28.2	103	26.7	45
2008	100	15	52.6	78	29.3	41
2009	-	-	100	20	80.6	31
Total	66.7	66	36.7	286	41	144

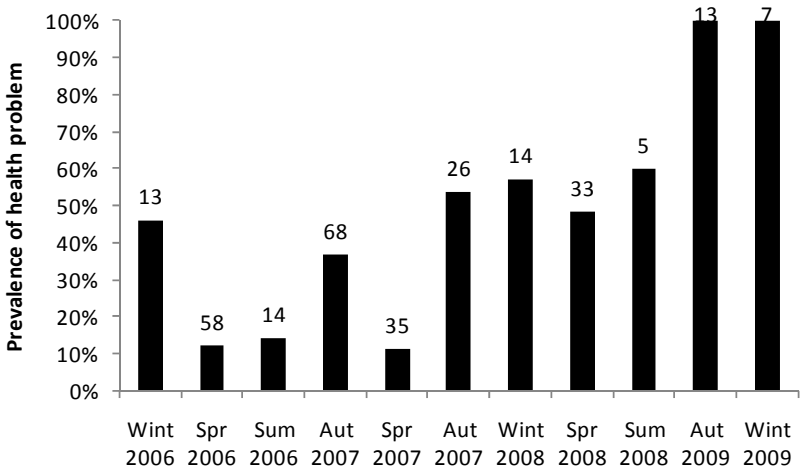

Figure 3.3 Prevalence of health problems in Keninup grouped by season within each year of the study. Numbers above bars indicate total sample size.

Table 3.12 Spearman's rho correlation coefficients between health problems, population abundance and decline estimates.*

		TS	AAD TS	APD TS	AD TS peak	PD TS peak
AAD TS	r_s	0.399				
	Sig. (1-tailed)	0.000				
	N	519				
APD TS	r_s	0.356	0.905			
	Sig. (1-tailed)	0.000	0.000			
	N	519	519			
AD TS peak	r_s	0.531	0.327	0.267		
	Sig. (1-tailed)	0.000	0.000	0.000		
	N	544	519	519		
PD TS peak	r_s	0.538	0.308	0.259	0.995	
	Sig. (1-tailed)	0.000	0.000	0.000	0.000	
	N	544	519	519	544	
Health problem	r_s	-0.213	-0.343	-0.302	-0.093	-0.075
	Sig. (1-tailed)	0.000	0.000	0.000	0.015	0.040
	N	557	519	519	544	544

r_s, Spearman's rho correlation coefficients; TS, trapping success; AAD TS, annualised absolute difference in TS; APD TS, annualised percentage difference in TS; AD TS peak, absolute difference in TS from peak; PD TS peak percentage difference in TS from peak. In bold significant p-values after Benjamini-Hochberg correction (Benjamini & Hochberg 1995). *Note that rates and extent of the decline have negative values when the decline is occurring and stabilise around zero when the population reaches a low density.

3.4 Discussion

3.4.1 Biometrics

A thorough analysis of the biometric differences between Karakamia and Upper Warren is beyond the scope of this study, since the data were simply used to determine a biometric index for individuals, and establish a minimum threshold of "good" body condition. Nevertheless, it is important to note the lack of difference in the average biometric index in Karakamia between males and females, when a significant difference was present in Upper Warren in both subadult and adult age classes. The extremely high population density of woylies in Karakamia (free of introduced predators) and the subsequent limitation of resources of the sanctuary best account for these population differences. Seasonal versus year-round reproduction, lower body weight at Karakamia and significant differences in the diet are also interpreted as evidence of a population responding to a limitation of food at Karakamia (DEC Science Division 2008a; K. Zosky personal communication).

3.4.2 Reference ranges

The reference ranges calculated showed that woylies have a higher concentration of RBC accompanied by lower MCV, MCH and CHCM when compared to the published reference ranges of other macropods (Clark 2004; see Vogelnest & Portas 2008 for a review). These findings are also consistent with what was observed in a preliminary woylie haematological investigation (Clark 2007). On the other hand, the leukocyte panel is broadly similar to other

macropods (Clark 2004; Vogelnest & Portas 2008). In many marsupials, lymphocytes are the most abundant leukocyte in the peripheral blood (Clark 2004) but this is not necessarily the case in macropods (Vogelnest & Portas 2008). It is interesting to note that lymphocyte concentrations showed a significant difference between the two geographic locations (see below). In Karakamia neutrophils are evidently the most common cell type, while in Upper Warren lymphocytes and neutrophils are present in comparable numbers. Several factors could be responsible for this discrepancy and are discussed further below (see Section 3.4.3, 3.4.4).

The reference ranges presented here were used to assist the identification of sick animals and are the best data available to date. Nevertheless, the analysis may be further improved and refined by including further disease data. For example, piroplasms and trypanosomes were found in the studied woylie populations (Clark & Spencer 2007; Smith et al. 2008a) and it would have been appropriate to remove positive animals from the dataset, but unfortunately, identification of positive animals was not available at the time this analysis was carried out.

3.4.3 Location and gender

Differences between genders in the RBC count are consistent with that found in other marsupials: an increased erythrocyte concentration was found in male allied rock-wallabies, *Petrogale assimilis* (Spencer & Speare 1992), greater gliders, *Petauroides volans* (Viggers & Lindenmayer 2001), common and mountain brushtail possums, *Trichosurus vulpecula*; *T. caninum*. (Presidente & Correa 1981; Viggers & Lindenmayer 1996) and, only in winter, in Tammar wallabies, *Macropus eugenii* (McKenzie et al. 2002). In contrast to what was found in this health investigation, no differences were reported in leukocyte parameters between

genders in previous marsupial haematological studies (Presidente & Correa 1981; Spencer & Speare 1992; Viggers & Lindenmayer 1996; McKenzie et al. 2002). The differences between woylie genders in the leukocyte panel could be related to different hormonal profiles and behavioural characteristics, as well as an increased susceptibility of one gender to specific pathogens (see Section 3.4.4). Nevertheless, based on changes in the haematological parameters, it would appear that these differences do not pose a greater risk for the general health of one gender over the other (i.e. there were no significant Odds Ratios).

It is difficult to discriminate whether the dissimilarities between Karakamia and Upper Warren in the leukocyte panel is due to physiological differences or whether it is an artefact of the slightly different handling techniques used in the two locations. Excitement, fear and pain can increase neutrophil and lymphocyte concentrations in marsupials (Clark 2004). In Upper Warren, animals were bled immediately after clearing the animals from the traps, while in Karakamia animals were removed from traps during the evening and kept in bags until early in the morning when blood samples were collected. It is possible that woylies at Karakamia had a chance to settle down before being bled, resulting in a lower lymphocyte count. On the other hand, the hypothesis that individuals in the two locations are exposed to different pathogens cannot be discounted. Additionally, the different proportion of animals showing lymphocytosis between the two populations in Upper Warren (where the same trapping methodology was used) suggests that at least part of these differences are due to different immune system stimulations, rather than being only a sampling artefact. The finding that the prevalence of gastro-intestinal parasites in woylies from the Upper Warren was significantly higher than those from Karakamia (DEC Science Division 2008a), further supports this hypothesis.

3.4.4 Seasons

Limited studies have been carried out on the effect of season on haematological parameters of marsupial species, particularly among macropods. The few published studies have small numbers of animals sampled within each sex for each season, restricting the statistical power and the numbers of possible analyses. For these reasons a comparison and identification of common patterns of seasonal changes of haematological parameters may be difficult. Additionally, the fact that these studies were carried out in different climatic regions further challenges the investigation on the effect of weather conditions on the physiology of free-range animals. Lastly, the lack of repeated surveys over several continuous years adds another difficulty to correctly differentiating seasonal discrepancies from stochastic variations. The results presented here are not immune to these issues. In spite of the fact that this study spanned over almost four years, due to the nature of the decline, the number of samples collected were not sufficient to enable a statistically meaningful comparison of the influence of season between different years. Consequently, it was impossible to differentiate between possible fluctuations between years as opposed to variation between seasons. Nevertheless, changes in the erythron panel were in line with the described seasonal haematological changes in Tammar wallabies (McKenzie et al. 2002) and mountain brushtail possums (Barnett et al. 1979). Similar PCV changes in quokkas (*Setonix brachyurus*) and HGB in Euros (*Macropus robustus*) have also been reported and it has been inferred that nutrition was responsible for these seasonal fluctuations (Ealey & Main 1967; Shield 1971). Likewise, water and food quantity and quality could probably explain these changes in the Upper Warren populations too. In light of this, it is worth noting that differences in the diet were also found between Warrup and the north-eastern blocks (Keninup and Balban. K. Zosky personal communication), with Warrup showing a greater variability in food sources, especially in autumn, than the other two blocks. Nevertheless, no quantitative assessment of the relationship between the nutritional composition of diets and the haematological profiles has been carried out and

biometric index differences would appear to conflict with this hypothesis. No seasonal changes in body conditions were found and woylies in Kingston were in slightly better condition than those from Perup, which is surprising if the seasonal fluctuations (and the higher risk of woylies in Kingston being anaemic) were truly associated with a reduced intake of nutrients. Parasite-induced anaemia (e.g. haemo- or endo-parasites) is expected to be more pronounced in the warmer months and this potentially could explain the seasonal haematological changes detected in this study.

The interpretation of leukocyte variation over the four seasons presented a challenge. Additionally, the little variance that was explained by seasons in the multiple regression analyses indicated that other factors are also responsible for these haematological changes. None of the differences found in this study were identified in the Tammar wallaby (McKenzie et al. 2002). Fluctuation of WBC counts were evident in allied rock-wallabies (Spencer & Speare 1992), however, the complexity of the variation in rock-wallabies and the significant climatic differences of the study location (a tropical weather pattern where summer is during the rainy season in Queensland) make it difficult to infer whether there are similarities between the present study and that of Spencer and Speare (1992).

Stress associated with extreme temperatures was suggested as a possible mechanism responsible for changes in the concentration of lymphocytes (Baker & Gemmell 1999). The lack of information on stress-hormones, such as cortisol, hampered the possibility of investigating this relationship in woylies. Nevertheless, it would be surprising that temperature changes were responsible for such erratic fluctuations (i.e. a more continuous and smooth transaction between seasons would be expected). Besides, the lowest lymphocyte counts were recorded in winter and summer, in contrast to the study by Baker and Gemmell (1999).

There is no evidence of leukocyte differences between seasons in other studies of marsupials species (e.g. Ealey & Main 1967; Shield 1971; Barnett et al. 1979; Presidente & Correa 1981) except for changes associated with the breeding season in male dasyurids (e.g. Cheal et al. 1976; Bradley 1990). The only exception is the report of an increased concentration of eosinophils in autumn in mountain brushtail possums (Viggers & Lindenmayer 1996). Changes in the eosinophil counts could be explained by an increased exposure/activity of parasites and vectors or migration of larvae in spring. Males of various mammalian species are known to be more prone to heavier infestations of parasites (Wilson et al. 2002). A gender bias in the parasite prevalence or burden could explain why, in woylies, these haematological alterations are evident only in males.

3.4.5 Patterns with respect to the decline and population abundance

Several analyses were undertaken in an attempt to identify patterns between woylie general health and demographic dynamics, but no clear evidence was found of positive association between these factors. For example, it is difficult to discriminate whether the marginal differences between haematological profiles before and during the decline in Keninup are a consequence of "normal" environmental fluctuations or early haematological changes of debilitating conditions. In the first case ("normal" environmental fluctuations), these changes could be simply associated with differences in the availability of food resources or parasite presence, load and/or within-host migration (Kerr 2002). In the second case (debilitating conditions), animals suffering severe clinical disease would be unlikely to be re-trapped, because the survival of these individuals would be greatly reduced making it almost impossible to provide evidence that this was the case. Additionally it was not possible to control for seasonality because of limited sample size.

Similarly, when considering the relationships between haematological variables and measurements of decline, there is not a clear pattern emerging with respect to the decline. For example, there is no consistent trend between the two genders or between populations (Tables 3.9-3.10). This suggests that there is not a causal relationship between the decline and changes in blood parameters. Moreover, Speaman's rho values were always small. Consequently, even though some correlations were statistically significant, these are likely to have little biological meaning.

An important source of bias could be generated by field operators. With the progress of the decline, field operators could have possibly paid more attention to the clinical examination of trapped woylies. Many field operators were involved in the data collection and, notwithstanding the efforts through training sessions to equalise the grading systems used, a degree of subjectivity in the description of the lesions as well as data quality is unavoidable. However, most of the health problems were recorded at the initial phase of the decline and the least number of health problems were recorded when the populations stabilised around 10% TS (Wayne 2006), as opposed to what would be expected if a strong operator-bias was present.

3.4.6 Future development and research

Several constraints limited the power of the present investigation. The initial intention of the WRCP was to carry out disease and biological investigations on the same subset of samples in order to enable a much more powerful analysis and facilitate the interpretation of results. Unfortunately this was not achieved effectively. A more carefully planned and designed effort

from the outset would have required human and financial resources that were not available. Furthermore, field observations were not quantified and codified prior to the beginning of this research project, but were recorded as qualitative comments, limiting the resolution of the analysis.

Further studies are recommended in order to improve this investigation should include the use of stored samples (sera, blood clots and faecal samples), as well as the disease data generated by collaborators of WCRP, in order to have a more comprehensive set of detailed information on a cohort of individuals.

All the analyses carried out in this study required independent samples, so multiple data entries from the same individuals were not considered. Whilst time constraints did not allow for this type of analytical study, such analyses could be informative and they are recommended. For example, it is possible to examine changes in the general health of the same individuals over time and investigate whether these changes influence the recapture probabilities (i.e. estimate of longevity).

The results of field health examinations were coded as a dichotomous variable (presence-absence). However, it may be possible to retrieve a greater level of detail from the qualitative comments recorded and enable a more thorough investigation of the effect of the physical problem reported on the woylie haematology and fitness.

Including the ID of the operator (the person who collected the data in the field) in the dataset could also help unravel possible human-caused bias.

A formal analysis of the relationship between rainfall and diet versus woylie general health would improve our understanding on the role of these environmental variables and facilitate the correct interpretation of differences between populations and seasons.

Finally, assessing demographic and body condition data from sympatric species might provide important information. For example, if a strong association between the conditions of more species was present, then a common environmental cause would be more likely rather than a species-specific factor.

3.5 Conclusions

Several aims associated with the analysis of haematological and body condition data were effectively achieved and this study represents a further contribution towards the identification of the cause of the woylie decline by providing important baseline data to evaluate woylie health and facilitate the interpretation of results of currently ongoing and future disease investigation of this species, as well as by providing recommendations regarding the direction of future research efforts for woylie conservation.

3.5.1 Haematological reference ranges

Haematological reference ranges were established and were used in the process of identifying unhealthy individuals. When other disease data become available from the currently ongoing research on woylie pathogens, the criteria used to determine “healthy” woylies should be revised and this will further refine the analysis. Despite the above mentioned limitations, the

sample size is among the largest ever used to establish haematological reference ranges in macropods and more generally, in marsupials (Melrose et al. 1987; Haynes & Skidmore 1991; Spencer & Speare 1992; Svensson et al. 1998; McKenzie et al. 2002; Clark 2004; Wicks & Clark 2005; Clark & Spencer 2006; Bennett et al. 2007; Reiss et al. 2008; Vogelnest & Portas 2008).

3.5.2 Differences in haematological parameters between locations (Upper Warren and Karakamia), populations (Kingston and Perup), gender and seasons

Several differences were detected. Leukocyte concentrations were greater in woylies from the Upper Warren compared with those from Karakamia. WBC and lymphocyte counts were greater in males than in females in Upper Warren. Differences between populations were responsible for the greatest amount of RBC variance (18.4%) in the multiple regression model. The erythron panel showed a declining pattern towards warmer months that was moderately explained by seasonality. The low amounts of variance due to the seasonal variation indicated that other variables are also responsible for such changes, most likely nutrition and rain fall.

The large general fluctuations in the leukocyte panel (mainly involving WBC, lymphocyte and eosinophil concentrations) were only partly associated with seasonality (4.1% of the variance of eosinophil concentration). Associations with commonly found pathogens (i.e. endo-, ecto- and haemo-parasites) should be investigated to determine the most likely cause of such variations.

3.5.3 Associations between changes in the general health of woylies and demographic dynamics

Rigorously assessing whether changes in health (by field examination and haematology) are associated with the woylie declines has been substantially limited by several fundamental factors including, sample sizes within cohorts, robust quantification of gross signs of poor health, and the complexity of multiple factors influencing diagnostic parameters (e.g. space, time, gender, age, diet, breeding, etc). Nevertheless, the increased risk of being lymphocytotic and the higher prevalence of observed health problems associated with animals in Perup, which includes two forest blocks that underwent a decline during sample collection, merit additional data analysis (as suggested above) and disease investigation. In western ringtail possums (*Pseudocheirus occidentalis*) increased WBC counts was associated with reduced survival (J Clarke[6], unpublished data), indicating that this finding in woylies could be relevant.

3.5.4 Assessment of geographic and demographic factors

Important differences were associated with the geographic location. Namely, animals in Kingston had an increased risk of being anaemic and animals in Perup were more likely to be lymphocytotic. It was not possible to identify the causes of these differences, but these results provide important clinical indications that will facilitate the interpretation of the results of other disease investigations.

[6] Clarke, J. R. Unpublished. Translocation Outcomes for the Western Ringtail Possum (*Pseudocheirus occidentalis*) in the Presence of the Common Brushtail Possum (*Trichosurus vulpecula*): Health, Survivorship and Habitat Use. Ph.D. thesis. School of School of Veterinary and Biomedical Sciences. Murdoch University, Murdoch.

Population abundance (calculated as TS) did not appear to influence haematological parameters with the possible exception of lymphocyte concentrations in Perup.

Based on the overall results of this health assessment and the outcome of the disease risk analysis (Chapter 2), it was considered adequate to progress with the investigation of a selected subset of diseases that were ranked as high priority and the results of these investigations are outlined in the following chapter (Chapter 4).

4 Virological investigation in declining woylie populations

4.1 Introduction

Diseases can regulate the demography of wildlife species and represent, by themselves or in association with other factors, a potential cause of decline (Caughley & Gunn 1996; Daszak & Cunningham 1999; Daszak et al. 2000; Aguirre et al. 2002). Emerging infectious diseases in wildlife are reported world wide and include a variety of pathogens such as viruses, parasites and protozoa (Daszak et al. 2000; Daszak et al. 2001). In Australia various native species are threatened by several diseases (Bunn & Woods 2005; Kirkland 2005; Skerratt 2005; Spratt 2005a; Symonds 2005) with most of these having viral aetiology, including Orbivirus and Herpesvirus (Kirkland 2005).

The determination of the presence of a pathogen in a population and its prevalence is the first step in a disease investigation (Artois et al. 2001). This allows the prioritization of future research in order to quantify the effects of specific pathogens on the population level of a particular wildlife species.

In woylies, a virological investigation was conducted to determine if an immune response to Macropod Herpesvirus (MaHV 1 and 2), Encephalomyocarditis virus (EMCV) and Orbivirus (Wallal and Warrego serogroups) was present. Reasons for the selection of these viruses are discussed in Chapter 2.

Herpesviruses are viruses with a single molecule of linear, double-stranded DNA with an envelope and icosahedral symmetry of the capsid. Herpesvirus virions are unstable in the environment and direct contact, or close proximity, between individuals is necessary to spread the infection. Macropod Herpesvirus belongs to the *alphaherpesvirinae* subfamily. Members of this subfamily, including MaHV-1 and 2 (Guliani et al. 1999), generally spread rapidly and establish a latent infection in the ganglia (Quinn et al. 2002). As with other herpesviruses, MaHV can reactivate as a result of immune suppression (Guliani et al. 1999). Herpesviruses are usually species-specific and highly adapted to their hosts and the hypothesis of co-evolution of these viruses with their hosts is well accepted (McGeoch et al. 1995; Mahony et al. 1999). However, the phylogenetic relationship of MaHV 1 and 2 with other viruses of the subfamily would appear to contradict the co-evolution theory (Mahony et al. 1999). Yet in spite of this only macropod species have been found to be susceptible to infection with MaHV, for example, inoculation of MaHV-1 failed to establish a systemic infection in the brushtail possum (*Trichosurus vulpecula*) (Zheng et al. 2004). Recently, a third species of MaHV was discovered in eastern grey kangaroos (*Macropus giganteus*) (MaHV-3, Smith et al. 2008b). This virus was identified in a captive colony in the United States and it was not available for testing; consequently, antibodies against this MaHV virus were not assessed in this study.

The encephalomyocarditis virus is a single molecule of linear, single-stranded (positive-sense) RNA, with icosahedral symmetry of the capsid and without an envelope. It belongs to the *Picornaviridae* and virions are resistant in the environment. The virus is shed in the faeces and urine of rodents that are the natural hosts. The infection develops in a few days and the virus can replicate in a broad spectrum of hosts including primates, pigs, rodents and marsupials (Reddacliff et al. 1997; McLelland et al. 2001; Quinn et al. 2002).

Orbivirus is a genus of the family *Reoviridae*. The genome of virions of this family contains ten to 12 segments of linear, double-stranded RNA. The capsid has an icosahedral symmetry and virus particles do not contain an envelope. Viruses of the genus *Orbivirus* are generally transmitted by arthropods, particularly *Culicoides* spp..

Clinical conditions associated with the infections of these viruses in marsupials are described in Chapter 2.

4.2 Materials and Methods

Woylies were trapped using a standard technique in the Upper Warren and Karakamia sanctuary (Figure 1.2). Blood samples were collected from the lateral tail vein via a 23 or 25 gauge needle in a 2.5 or 3 ml syringe and then transferred into plain tubes. After blood clots were formed, samples were chilled in an esky with ice in the field. Upon return from the field, blood samples were centrifuged as soon as possible (but never later than eight hours after collection), and sera separated and frozen at -20°C until tested.

The selection of samples to be tested for specific tests required careful determination given the small amount of serum obtained from animals sampled and the limited number of samples available, especially from low density forest blocks. Under the hypothesis that the virus tested was the cause of the decline, two main criteria were used to select the samples:

1. The pathogen should be present in all forest blocks because it was theorised that declines within each block were not independent (Wayne 2006; Wayne et al. 2008b).

2. It would be more likely to detect seropositive individuals in the population after the decline because survivors would be likely to have immunity (Thompson et al. 1992; Roelke-Parker et al. 1996; Tompkins et al. 2002; Härkönen et al. 2006; Thrusfield 2007).

Consequently, only post-decline samples from Balban (Figure 1.3, Table 4.1) were tested for EMCV and the two *Orbivirus* serogroups. Woylie from a broader geographical area were tested for MaHV including the control population (Karakamia) and 15 sampled pre-decline in Keninup (Figure 1.3, Table 4.1). When available, multiple samples collected at different time points from the same individual were also tested (Table 4.1).

The maximum possible prevalence (i.e. power analysis) with 90 and 95% confidence was calculated following the methods of Cannon and Roe (1982), using Episcope 2.0 (Thrusfield et al. 2001) with an estimated population size of 1,000 (Groom 2010).

Virus neutralization tests (VNTs), for MaHV were conducted with a slightly different protocol from the other viruses. All sera samples were heat inactivated at 56°C for 30 minutes.

For MaHV, VNTs were carried out as follows: 10 µL of sera were diluted in 20 µL of phosphate buffer solution (PBS) and mixed with 10 µL MaHV-1 control virus and incubated at 37°C for one hour. The virus/serum mixture was then added, in 24 well plates (Nunc, Roskilde, Denmark), to the monolayer of PtK (*Potorous tridactylus* kidney cells) previously washed from the media with sterile PBS. After the addition of 1mL of maintenance media, the cell culture plates were incubated at 37°C in an atmosphere containing 5% CO_2. A known positive and negative control was included in each plate. Plates were observed daily for presence of cytopathic effect (CPE) in the monolayer until it was degraded and recognition of CPE impossible.

The VNTs for EMCV and the two *Orbivirus* serogroups were carried out in 96 well plates (Cooke Engineering Co., Alexandria, VA) as follows:

A control plate was set for each test which included a positive and negative control, a dilution series of the positive control, cell control wells with no added virus or serum, a virus titration, and a working strength titration. Additionally, sample sera, after inactivation at 56°C for 30 minutes, were diluted with foetal calf serum to 1:4 and an aliquot was added to a well with no virus to check for toxic effects on the cell cultures. Finally, a dilution series (from 1:4 to 1:32) of sample serum was mixed with a constant virus concentration and baby hamster kidney (EMCV) or BSR (*Orbivirus*) cells suspended in Dulbeccos' modified eagle medium (DMEM; EMCV) or a 1:1 mix of DMEM and Basal medium eagle (*Orbivirus*), and incubated at 37°C in an atmosphere containing 5% CO_2. Within 24-48 hours of incubation, cell cultures were checked for proper attachment to the bottom of the well and at the fifth day for CPE.

4.3 Results

The CPE caused by MaHV-1 was already evident at the second day of incubation and all samples had evidence of CPE after 72 hours. Complete degradation of the monolayer was apparent after 5-7 days. No CPE was observed in any of the negative control wells.

Similarly, CPE was observed in all samples tested for EMCV, Warrego and Wallal serogroups, except for two samples at 1:4 and 1:8 when tested for the Wallal serogroup. However, that particular test was under challenged and when it was repeated the samples were positive only at 1:4 with a still slightly under challenged assay. Unfortunately, the lack of sera prevented further repeats of the test. Nevertheless, it was considered to be negative and the neutralisation present was assumed to be non-specific (Reddacliff et al. 1999).

Table 4.1. Summary of samples tested and maximum possible prevalence.

Locations	Balban				Keninup	Warrup	Karakamia
Virus	EMCV	WAR	WAL	MaHV	MaHV	MaHV	MaHV
Pre-decline	0	0	0	0	15	0	NA
Post-decline	66	44	38	50	48	49	NA
Total individual tested	66	44	38	50	63	49	38
Total individual with multiple samples	16	6	11	11	9	2	0
Total samples	86	51	51	65	72	51	38
Pr 90%Conf	3.4%	5.0%	5.8%	4.4%	3.5%	4.5%	5.8%
Pr 95%Conf	4.3%	6.5%	7.5%	5.7%	4.5%	5.8%	7.4%

EMCV: Encephalomyocarditis virus. WAR: Warrego serogroup. WAL: Wallal serogroups. MaHV: Macropod herpesvirus. Pr 90%-95%Conf: maximum possible prevalence at 90% and 95% confidence, based on total number of individuals tested and assuming a population size of 1000 animals.

4.4 Discussion

Overall, there is no serological evidence of any of the tested viruses affecting the populations. However, due to the limited sample size, it was not possible to exclude infection from these viruses but only to exclude a seroprevalence above the maximum detectable prevalence (Table 4.1).

There is direct evidence that woylies are susceptible to MaHV, as infection in a captive colony of woylies housed at the Perth Zoo resulted in a case fatality rate of 100% over a period of one week (Dickson et al. 1980). However, there is no information available about possible morbidity and fatality rates caused by MaHV, or the other viruses tested in this study, in wild woylie populations to judge whether disease caused by one of these viruses may be responsible for the declines and result in post-decline prevalences lower than the maximum

detectable prevalence (3.4-7.5%, Table 4.1). Additionally, it could be expected that, after an epidemic with a very high fatality rate, as would be expected with MaHV, only a very small fraction, if any, of the population would have detectable antibody levels, despite the significant regulation that the disease would impose on the population demography. In such a scenario, where susceptible animals were exposed to a highly fatal disease, all, or almost all, infected animals would die and therefore it would not be expected that the disease could be detected (serologically) unless sick animals were trapped prior to death. A similar situation was modelled in fox populations where, assuming that a viral respiratory infection causes the death of 50% of infected animals, a prevalence of 0.18% would be sufficient to regulate the demography of the populations (Anderson 1995).

The results of this virological investigation contributed to the baseline data needed to improve the understanding of the population dynamics of woylies and to better assess risks for management of, as well as translocation among, these populations as well as the establishment of new populations. Regardless of whether these viruses are present in the populations at a lower prevalence than the maximum detectable level, or whether they are completely absent, it can be assumed that a substantial proportion of individuals have not been exposed to these viruses and therefore these populations may be particularly susceptible. Anthropogenic introduction of these pathogens in woylie populations must be avoided and preventive measures adopted. While current hygiene protocols adopted by DEC are exhaustive (Chapman et al. 2008) and transmission of these viruses is unlikely through movements between sites by people and equipment (as long as the protocols are followed meticulously), management protocols to prevent the spread of these diseases when dealing with live animals are established on a case by case basis (Chapman et al. 2008). These procedures should include quarantine and health screening. Circumstances where such protocols are particularly desirable are reported below.

Quarantine and viral serological screening is recommended before wild rehabilitated woylies or individuals sourced through a captive breeding program are released back into the wild, if these woylies were housed in direct contact or in proximity with other macropods. Clinical and latent infection with MaHV has been widely demonstrated in other species of macropods, especially in captive populations (Webber & Whalley 1978; Finnie 1980; Kerr et al. 1981), and it could have devastating consequences if introduced into woylie populations. When infection with EMCV is potentially possible (for example, captive groups with known presence of rodents in enclosures), the establishment of quarantine periods (associated with good rodent control in the quarantine facility) before releasing woylies into the wild is recommended, and can be considered sufficient to prevent the introduction of this pathogen into the target population, since, except in rodents, infection with EMCV generally causes sudden death shortly after infection (Reddacliff et al. 1997; McLelland et al. 2001; Fowler & Miller 2003; Jackson 2003). It is more difficult to put in place a control protocol for infections from *Orbivirus*, since these viruses are transmitted by arthropods, making the exposure to the viruses a possible eventuality, especially in areas were Wallal and Warrego viruses are known to occur (e.g. south west Western Australia, Hooper 1999; Hooper et al. 1999). However, infection with Wallal and Warrego viruses results in clinical signs in macropods (Fowler & Miller 2003; Jackson 2003), and it would be expected that these signs would be evident during the quarantine period.

Finally, wild caught animals found positive for any of the investigated viruses (EMCV, Wallal or Warrego serogroups or MaHV), should not be translocated to different sites for supplementation of wild populations or included in captive breeding programs.

5 Capturing genetic information using non-target species markers in a species that has undergone a population crash.

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5.1 Abstract

Species conservation has relied on the enormous potential of information that arises from field, laboratory and other tools. When using molecular-based tools, the technology involves a considerable effort to develop, both in resources and time. A long held practice has been to utilise pre-existing primers developed for other closely related species to evaluate conservation questions. In this study, we present a practical approach on how to utilise pre-existing microsatellite markers in bettong and potoroo species. This information is relevant prior to, during and after a species crash and the approach we describe could be particularly appropriate when there is an immediate need to retrieve a knowledge-base in order to support management decisions. We determined that cross-species amplification success of

microsatellite markers is inversely related to evolutionary distance of the source species although their polymorphism is not. A 'priority-list' of potential markers for potoroids is given for future conservation genetic studies.

Additional keywords: cross species amplification, population crash, potoroid, macropodid, microsatellite, *Bettongia penicillata*.

5.2 Introduction

Molecular ecology is an increasingly important and complementary part of classical ecological studies. This is because these techniques offer elegant approaches to improve our understanding of the ecology of a species that are difficult to obtain in the field with other methods (Sunnucks 2000). Such information could be vital when it comes to actively manage a species facing an impending threat of extinction. However, population genetic analysis is subject to the availability of tools that offer sufficient resolution to answer the questions being addressed. In addition, support for management decisions needs to have enough time and resources available to properly develop the best means to carry out these tasks. Here, we provide a guide on how to condense the selection process when choosing suitable markers for population genetic investigations in potoroids using the woylie or brush-tailed bettong (*Bettongia penicillata*) as a model. This species has experienced a dramatic population crash within a very short period of time, with losses of up to 93% within the last few years. As an example, *B. penicillata* populations in the Upper Warren region of Western Australia had an estimated population size of 20,000 individuals in 2001 but at the beginning of 2009, it was thought to be less than 1000 animals (Dr A. Wayne, DEC Science division, Manjimup).

Quantifying genetic changes and the consequences of catastrophic population decline is important in any conservation study, and there are two variables that affect our capacity to detect this with reasonable statistical power. One is a sufficient sample size, and the other is using a sufficient number of independent loci to make a meaningful inference from the samples available at the time of investigation. The trade-off is that employing a high number of loci may overcome the lack of large sample size, which is often the case in wildlife studies because of practical or financial limitations, a combination of both or, in some instances, simply only a small wild population can be sampled for genetic analysis. Microsatellite loci have been recognised as a useful genetic marker for common conservation genetic applications (Sunnucks 2000), either on their own or coupled with other genetic markers such as mitochondrial DNA. The development of novel primers involves substantial resources and financial costs although recent innovation in molecular technology, such as high-throughput sequencing technologies, substantially reduced these requirements compared to former methods (Abdelkrim *et al.* 2009). However, in the case of rapidly declining taxa, where management require a fast response (e.g. with *B. penicillata*), then the simplest immediate measure remains the use of existing and proven polymorphic markers. All available data suggested that the more polymorphic markers are best for cross species work (e.g. Zenger et al. 2003b; Donaldson & Vercoe 2008). As such, we chose 32 of the most polymorphic loci from a broad range of macropodoid species, including all published markers developed in the Potoroidae, *Petrogale xanthopus* (yellow-footed rock-wallaby) and *P. assimilis* (allied rock-wallaby), and due to practical limitation, only a subset of primer pairs developed for *Macropus* species. Additionally, the potential hidden presence of null alleles may require carefully evaluation by the researchers (Dakin & Avise 2004). To our knowledge, Zenger *et al* (2003b) is the only published study to describe cross-species use of microsatellite primers in macropodoids, but it provided insufficient information about the potential of those primers to

be used in bettongs and potoroos. In fact, only three primer sets, sourced by *B. tropica*, were tested in *Macropus eugenii* (tammar wallaby), and none from the genus *Potorous*. This issue was partly addressed by Donaldson and Vercoe (2008) who found no relationship between amplification success and genetic measure of evolutionary distance but it is likely that (as the authors acknowledge) the lack of correlation was due to the small number of loci tested. Additionally, they did not quantify the evolutionary distance among the species they tested, but considered equally distant genera within the same family, and families within the superfamily Macropodoidae. Moreover, most studies have utilised fewer than ten loci. These observations call for a better understanding of cross-species use of microsatellite primers in potoroids and our aim is that this study will facilitate future ecological and genetic investigations in bettongs and potoroos.

5.3 Materials and methods

5.3.1 Sample collection and DNA extraction

A total of 102 (presumably) unrelated adult *B. penicillata* were trapped using standard techniques in the Upper Warren region of the south-west of Western Australia (~34°9'S, 116°19'E). Pouch young were excluded from any analysis. Skin biopsies were taken and preserved in 70% Ethanol. DNA was extracted using a modified high-salt method (Miller *et al.* 1988) and the resulting DNA pellet was re-suspended in 1 × TE (50 mM Tris-base, 20 mM EDTA) at a final DNA concentration of ~50 ng/μl.

5.3.2 Microsatellites amplification

Thirty two primer pairs (originally developed for different macropodoid species; Table 5.1) were synthesised (Integrated DNA Technologies, Coralville, IA, USA) with a M13 tag (Schuelke 2000) and tested to assess their suitability using two different *B. penicillata* DNA extractions. 25µL PCRs were conducted in 1 × Reaction Buffer (Biotech), 1.5 mM $MgCl_2$, 0.1 mM Bovine Serum Albumin (BSA), 400 µM dNTPs, 0.4 µM of forward and reverse primer, 0.5 U of *Taq* polymerase (Tth Biotech) and 100 ng template DNA using a 'Touchdown' approach (initial denaturation at 95° C for 3 min, then 15 'Touchdown' cycles of: 95° C for 45 sec, 62-47° C for 30 sec, 72° C for 45 sec). The last cycle was followed by 30 cycles of: 95° C for 45 sec, 47° C for 30 sec, 72° C for 45 sec. This was followed by a final extension step at 72° C for 10 minutes. All the reactions that generated a visible product, with either of the two samples, on a 3% agarose gel were optimised further using a temperature gradient (from 47 to 62°C). The two temperatures that generated the most intense product on 3% agarose gel were used as annealing temperature to test for polymorphism and reproducibility using the M13 method described by Schuelke (2000) with eight adult *B. penicillata* DNA extractions using the following condition: 1 × Reaction Buffer (Biotech), 2.5 mM $MgCl_2$, 0.1 mM BSA, 320µM dNTPs, 0.16µM of FAM-M13 primer, 0.08µM of forward primer, 0.16µM of reverse primer, 0.5 U of *Taq* polymerase (Tth Biotech) and 100ng template DNA. PCR conditions included an initial denaturation at 95°C for 3 min, then 30 cycles of: 95°C for 30 sec, a variable annealing temperature depending on the specific temperatures (Table 5.2) for 45 sec, 72°C for 45 sec. This was followed by 8 cycles of: 95°C for 30 sec, 53°C for 45 sec, 72°C for 45 sec. Lastly, a final extension step at 72°C for 10 minutes was carried out. To minimise scoring errors and to ensure reproducible results, the same eight animals have been scored a minimum of five times.

Primer pairs that amplified polymorphic loci were fluorescently labelled (Appendix 1[7]) without the M13 tag. The reactions were then used in multiplex combinations (except for loci Pa593 and Y151 which were amplified using a single-plex approach, Appendix 1) to score 102 adult *B. penicillata* samples. DNA fragment analysis was carried out using a 5-dye system on an Applied Biosystems 3730 DNA Analyser (ABI systems, Melbourne). PCR product sizes were determined by co-running a size standard (Genescan™-500 LIZ™; Applied Biosystems, Melbourne) and fragments were scored with the aid of GeneMarker Software (SoftGenetics).

[7] Appendix 2 in this thesis.

Table 5.1 Details of the microsatellite loci tested with woylie (*Bettongia penicillata*) DNA extractions.

Locus	GenBank	PCR product		Polymorphic in the woylie		Reference
Bettongia tropica						
Bt64		Yes		Yes		(Pope *et al.* 2000)
Bt76		Yes		Yes		(Pope *et al.* 2000)
Bt80		Yes	100%	Yes	100%	(Pope *et al.* 2000)
Petrogale assimilis						
Pa55		Yes		No		(Spencer *et al.* 1995)
Pa297	U30634	Yes		No		(Spencer *et al.* 1995)
Pa385	U30632	No		No		(Spencer *et al.* 1995)
Pa593	U30633	Yes		Yes		(Spencer *et al.* 1995)
Pa595	U30635	No		No		(Spencer *et al.* 1995)
Pa597	U30636	Yes	60%	No	16.6%	(Spencer *et al.* 1995)
Petrogale xanthopus						
Y105		Yes		Yes		(Zenger *et al.* 2002)
Y112		Yes		Yes		(Zenger *et al.* 2002)
Y148		Yes		No		(Pope *et al.* 1996)
Y151		Yes		Yes		(Pope *et al.* 1996)
Y170		Yes		Yes		(Pope *et al.* 1996)
Y175		Yes		Yes		(Zenger *et al.* 2002)
Y76		Yes	100%	No	71.4%	(Pope *et al.* 1996)
Potorous longipedis						
Pl2	Y09050	Yes		Yes		(Luikart *et al.* 1997)
Pl3	Y09051	Yes		No		(Luikart *et al.* 1997)
Pl13	Y09052	No		No		(Luikart *et al.* 1997)
Pl18	Y09053	Yes		No		(Luikart *et al.* 1997)
Pl22	Y09054	No		No		(Luikart *et al.* 1997)
Pl26	Y09055	Yes	60%	Yes	30%	(Luikart *et al.* 1997)
Onychogalea fraenata						
B90		No		No		(Pope *et al.* 2000)
B123		No	0%	No	0%	(Zenger *et al.* 2002)
Macropus giganteus						
G31-1	AF322629	No	0%	No	0%	(Zenger & Cooper 2001b)
Macropus eugenii						
Me15	AF025909	Yes		No		(Taylor & Cooper 1998)
Me16	AF025910	Yes		No		(Taylor & Cooper 1998)
Me17	AF025911	Yes		No		(Taylor & Cooper 1998)
T17-2	AF326948	Yes		Yes		(Zenger & Cooper 2001a)
T31-1	AF326953	Yes		No		(Zenger & Cooper 2001a)
MeY01	DQ641481	Yes		No		(Macdonald *et al.* 2006)
MeY37	DQ641488	Yes	100%	No	14.3%	(Macdonald *et al.* 2006)
Total (% = average)		32	78.1%	12	37.5%	

Species are listed in increasing evolutionary distance from *B. penicillata*. GenBank = Genbank accession number; Yes/No, indicates that a PCR product was/wasn't produced. Percentages describe the proportion of loci that amplified and were polymorphic within each species, respectively.

5.3.3 Genetic and Statistical analysis

Genotypic data was initially manipulated using Microsoft® Excel 2002 SP3 (Microsoft Corporation). Quality of the data and early detection of null-alleles and allele drop-out was screened with MICRO-CHECKER (Van Oosterhout *et al.* 2004). Descriptive statistics, including the number of alleles (N_A) and expected heterozygosity (H_E) was calculated using GENALEX 6.2 (Peakall & Smouse 2006). We also calculated the Mean Fragment Length (MFL) and the allelic diversity ratio (a ratio of the number of alleles amplified in *B. penicillata* relative to the number of alleles in the source species), to measure the level of polymorphism, using Excel and, following Zenger *et al*. (2003b), compared these parameters with the source species as reported in Luikart *et al*. (1997), Zenger and Cooper (2001a) and Zenger *et al*. (2003b), with the non-parametric Wilcoxon signed-rank test. All statistical analyses were performed using SPSS v.15 (SPSS Inc.).

Partial sequences of cytochrome b gene were obtained from Genbank for *B. penicillata* (AY237238-9), *B. tropica* (AY237236-7), *Potorous longipes*: (AY237232-3, AY237248), *M. eugenii*: (AY237226). *M. giganteus* (AY099267, EF368023, MGU87137), *Onychogalea fraenata* (AY099278), *P. xanthopus* and *P. assimilis* (Mark Eldridge unpublished data.) and average pairwise evolutionary divergences were calculated with MEGA version 4 (Tamura *et al.* 2007) using the Kimura 2-parameter (Kimura 1980) as indication of evolutionary distances between *B. penicillata* and the source taxa. All positions containing gaps and missing data were eliminated from the dataset leaving a total of 398 sites in the final dataset.

5.4 Results

The microsatellite primers produced a PCR product for 25 (78%) of the 32 pairs tested, and 40% of loci were polymorphic when tested with the *B. penicillata* (Table 5.1). Null alleles were not detected. The average mean fragment length, number of alleles and expected heterozygosity were not significantly different in *B. penicillata* when compared with the source species (Table 5.2). The effect size was between medium and large (r=0.35-0.67), except for the MFL comparison with *P. xanthopus* (r=0.08), and it may have reduced the statistical power of these tests (Cohen 1988). The average N_A derived from primers developed for *P. xanthopus* (P= 0.043) was the only significantly different. This appears to be due to a single locus (Y151). When Y151 was removed, the difference was no longer significant (P=0.68). Size ranges overlapped with the source species. However, it was somewhat surprising that the locus Y151 showed large size variation compared with the source species, *P. xanthopus.* The percentage of loci that were polymorphic was inversely proportional to evolutionary distance (R^2=0.627, P=0.034, Fig. 5.1) while no relationship was evident when comparing the level of polymorphism (measured as allelic diversity ratio; R^2= 0.052, P>0.05).

Table 5.2 Details of the 12 microsatellite loci amplified in woylies (*Bettongia penicillata*) and comparison with source species.

Source species locus	Tm	Size range (bp)		MFL (bp)		N_A		H_E	
		source	woylie	source	woylie	source	woylie	source	woylie
Bettongia tropica									
Bt64	TD	146-196	152-204	173	180.25	12	18	0.852	0.888
Bt76	61	196-224	193-241	210	223.25	7	17	0.739	0.873
Bt80	61	183-199	180-200	189.33	187.28	5	10	0.7604	0.804
Mean				*190.78*	*196.9*	*8*	*15*	*0.784*	*0.855*
P				*0.285*		*0.1088*		*0.1088*	
Petrogale assimilis									
Pa593	57	131-159	100-130	148	116.71	12	15	0.826	0.896
Petrogale xanthopus									
Y105	61	244-258	223-251	251	233.39	8	11	0.775	0.766
Y112	61	171-195	183-235	180	209.65	6	21	0.677	0.913
Y151	57	179-205	174-336	194	231.37	9	29	0.787	0.913
Y170	TD	138-166	124-156	157	136.06	8	16	0.764	0.868
Y175	TD	267-299	254-286	287	266.67	12	14	0.801	0.883
Mean				*213.80*	*215.43*	*8.60*	*18.20*	*0.76*	*0.87*
P				*0.6858*		*0.0431*		*0.0796*	
Potorous longipes									
Pl2	61	150-160	130-154		143.80	4	10	0.648	0.693
Pl26	TD	164-184	155-161		157.03	3	4	0.335	0.620
Mean					*150.41*	*3.50*	*7.00*	*0.49*	*0.66*
P						*0.18*		*0.18*	
Macropus eugenii									
T17-2	TD	115-147	80-112		96.40	10	15	0.895	0.898

Species are listed in increasing evolutionary distance from *B. penicillata. T*m: Annealing temperature. MFL: Mean Fragment Length (n.a., data is missing for *Potorous longipes* and *Macropus eugenii* because not reported in the original publication). N_A: Number of alleles. H_E: Expected heterozygosity. TD: touchdown.

5.5 Discussion

This study demonstrated that from a total of 32 polymorphic macropodoid markers, 12 primer pairs were useful to quantify important population changes in a declining potoroid species. All 12 loci were highly polymorphic, making them extremely useful for conservation genetics purposes. The success of cross-species microsatellite amplification appeared to be inversely proportional to the genetic distance although it did not follow taxonomic organization as referred by Donaldson and Vercoe (2008), because genera within the same family and families within the Macropodoidae are not necessarily equally distant. This contrasts to the findings of Donaldson and Vercoe (2008), where no relationship was detected. The distance from *B. penicillata* is relatively large for all taxa except *B. tropica*. Even the genus *Potorous* has its most recent common ancestor (with *Bettongia*) around 20 Mya, while the genus *Bettongia* is as recent as 8 Mya (Westerman *et al.* 2004). However, it was unexpected that only two primer pairs originally developed for *P. longipes* would generate a high number of alleles and reproducible results in *B. penicillata*. It is possible that a limited number of mutations in critical (3'-) positions may compromise the efficiency of these primers. Additionally, the number of alleles in the source species was relatively small, suggesting that it is possible that these loci could be monomorphic in *B. penicillata*.

Cross-species performances (as measured by allelic diversity ratio) showed a lack of linear decrease, in contrast to the report by Zenger *et al* (2003b). However, the limited number of polymorphic loci amplified using primer pairs developed for *Macropus* species may have reduced the resolution of this analysis. Additionally, the high level of polymorphism, in *B. penicillata*, could have been influenced by the recent demographic history of the population.

In fact, the *B. penicillata* population in Upper Warren had (in the last 20 years) a relatively large census size (Start *et al.* 1998; Mawson 2004; Orell 2004; Wyre 2004) as a consequence of fox control measurements. This may have helped to prevent localised loss of rare alleles and to maintain a relatively high level of genetic variability. Equally, demographic histories of source species may have influenced their levels of genetic diversity. For example, the geographical extent of natural occurring populations of the endangered *P. longipes* are limited (Luikart *et al.* 1997) potentially causing a lower genetic diversity of what would be otherwise expected.

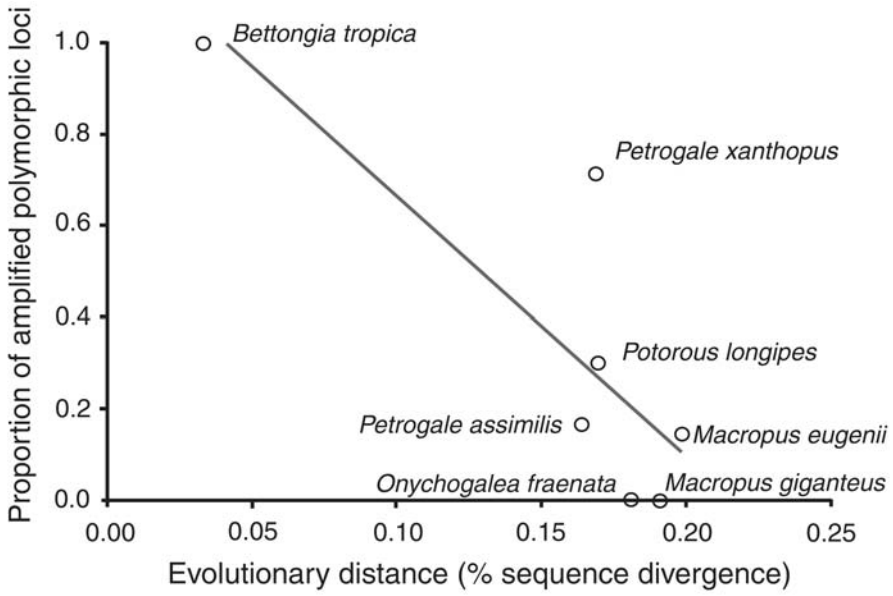

Figure 5.1 Amplification success of polymorphic microsatellite loci in woylies (Bettongia penicillata).

The X-axis displays evolutionary distance from *B. penicillata* based on percentage cytochrome *b* pairwise divergence (see Methods).

We believe that this study provides important information to those who are intending to investigate the genetic profile in bettongs and potoroos.

We identify a number of practical suggestions that arose from this study, some of which may aid those considering similar work.

1. The use of M13-tags (Schuelke 2000) dramatically reduced the cost of testing of a large number of markers.
2. We believe that the 12 markers that worked in *B. penicillata* are highly likely to be able to universally applicable in closely related species
3. From our (limited) data it is suggested that there is a 50% reduction in amplification succuss of polymorphic loci for every 1 million years of evolutionary distance from taxa.
4. We would recommend using markers from the closest available taxa as first choice.
5. If a larger number of loci are required, the results presented here provide a 'priority-list' of potential markers that may be used to genotype polymorphic loci for potoriones.
6. Primers that have produced a product may be used as a starting point to generate specie-specific primers without the necessity to continue with the resources required to build an entire species-specific genomic library or purchase expensive new technologies. The products could then be sequenced and the flanking regions could be re-designed giving rise to more efficient primers.

5.6 Acknowledgements

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6 Effects of habitat fragmentation on population structure and long distance gene flow in an endangered marsupial: the woylie.

This chapter is in review as an original article in the Journal of Zoology.

Citation: Pacioni, C., A. F. Wayne, and P. Spencer (Submitted). Effects of habitat fragmentation on population structure and long distance gene flow in an endangered marsupial: the woylie.

6.1 Abstract

A deep understanding of population structures and of the relationships among populations is fundamental to guarantee adequate management of endangered species. We used a molecular approach (12 microsatellite loci and mitochondrial DNA) to investigate these aspects in the woylie or brush-tailed bettong (*Bettongia penicillata ogilbyi*). Four distinct indigenous populations were identified in this study (i.e. Dryandra woodland and Tutanning Nature Reserve in the wheatbelt region and two discrete populations in the Upper Warren in the south-west forests of Western Australia). Additionally, previously undisclosed modern and

historical connections between these units became evident, such as the historical connection between populations at 150 km distance (Dryandra and Upper Warren) and the contemporary gene flow between the two populations in Upper Warren (up to 60 km). Genetic attributes of the four populations were analysed and the evidence of unique genetic material in each of these populations indicated that conservation effort should aim towards the preservation of all these units. Additionally, the lower genetic diversity of the woylie population in Tutanning Nature Reserve prompted the need for the investigation of factors that are limiting the demographic growth of this population. This study enhances not only our knowledge about the ecology of woylies, but also the genetic consequences of habitat fragmentation and reiterates the strength and pertinence of molecular techniques in similar investigations.

Keywords: Woylie, brush-tailed bettong, *Bettongia penicillata*, Potoroidae, microsatellites, mtDNA, population structure, connectivity.

6.2 Introduction

Our understanding of the ecology of a species is greatly improved by knowledge about its population structure and level of connection among units (Paetkau et al. 1995). Habitat fragmentation effects on population dynamics also need to be understood to thoroughly assess the risks posed by processes that could threaten the conservation of many species (e.g. reduced dispersal, smaller effective population size and increase inbreeding. Banks et al. 2005). The identification of management units and evolutionarily significant units (Moritz et al. 1994; Moritz 1999) is also fundamental to correctly manage endangered wild populations (Zenger et al. 2003a).

For example, knowledge of genetic attributes are needed for the effective recovery of woylie or brush-tailed bettong (*Bettongia penicillata ogilbyi*), which has recently been listed as critically endangered. So far it has declined by about 80% between 2000 and 2006 (Wayne et al. 2009). Less than 2000 individuals remain in the three localities where indigenous populations persist - Upper Warren region, Dryandra woodland and Tutanning Nature Reserve, all in south-western Australia (Fig. 6.1). The woylie continues to decline. It has been hypothesised that predators and/or a disease may be a concomitant cause if not the primary cause(s) of the decline based on available associative evidence (DEC Science Division 2008a).

Information on the population structure and movements between populations is important to assess the direct disease transmission risks and to help determine an effective conservation strategy for the species. This study focused on the extant indigenous woylie populations and used mitochondrial DNA (mtDNA) and microsatellite loci (MS) to: i) identify genetically distinct populations and possible sub structuring, ii) establish population relationships both historically and contemporarily, iii) determine the overall genetic variability and differences, within and among populations, iv) provide an indication of long term genetic viability and suggest management directions to prevent potential loss of unique genetic material.

Considering the small home ranges of the woylie (19.6-34.8 ha) and the short distance commonly observed in dispersal events (Sampson 1971; Christensen 1980), we would predict that the degree of genetic divergence among populations should be directly proportional to their geographic distance. The locations we describe have been effectively isolated from each other since the 1920s-1960s. Consequently, the three localities should represent at least three discrete populations. In the closely related northern bettong (*B. tropica*), populations at more than 12 km of distance were genetically distinct despite being geographically connected by

continuous habitat (Pope et al. 2000). Thus, it is possible that woylie populations are further structured within each of the three localities.

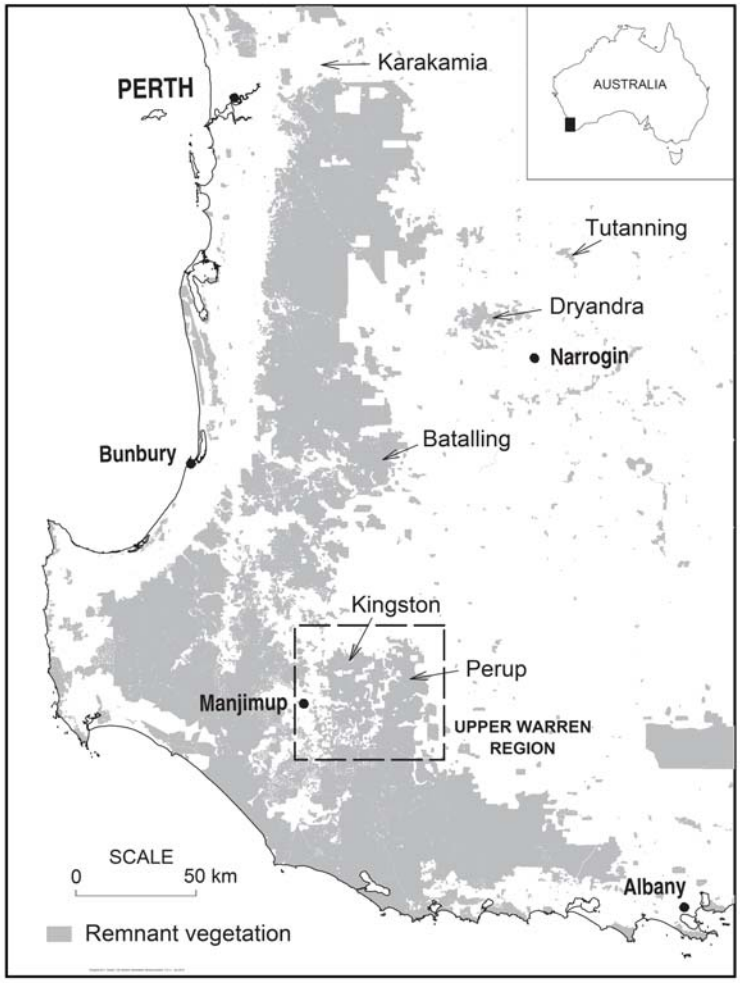

Figure 6.1 Important woylie populations (arrows) and towns (dots) in Western Australia.

6.3 Methods

6.3.1 Sample collection

A total of 231 tissue samples were collected between 2006-2008 (Table 6.1) from all three remaining indigenous (natural) woylie populations (Upper Warren in the south-west forests, Dryandra woodland (Dryandra) and Tutanning Nature Reserve (Tutanning) in the wheatbelt region of Western Australia (Fig. 6.1)). In Upper Warren, woylies were trapped using standard monitoring techniques from 11 forest blocks (Fig. 6.2) conducted by the DEC (Department of Environment and Conservation) using live-cage trapping transects (50 cages per transect spaced 200 m apart) (Orell 2004); no woylies were trapped in two of these areas where previously they had been abundant (Yackelup and Camelar). In the wheatbelt populations (Dryandra and Tutanning), in addition to a standardised transect, woylies were also trapped with opportunistic traps throughout the areas. Small tissue samples from the ear (skin biopsies) were stored in 70% ethanol.

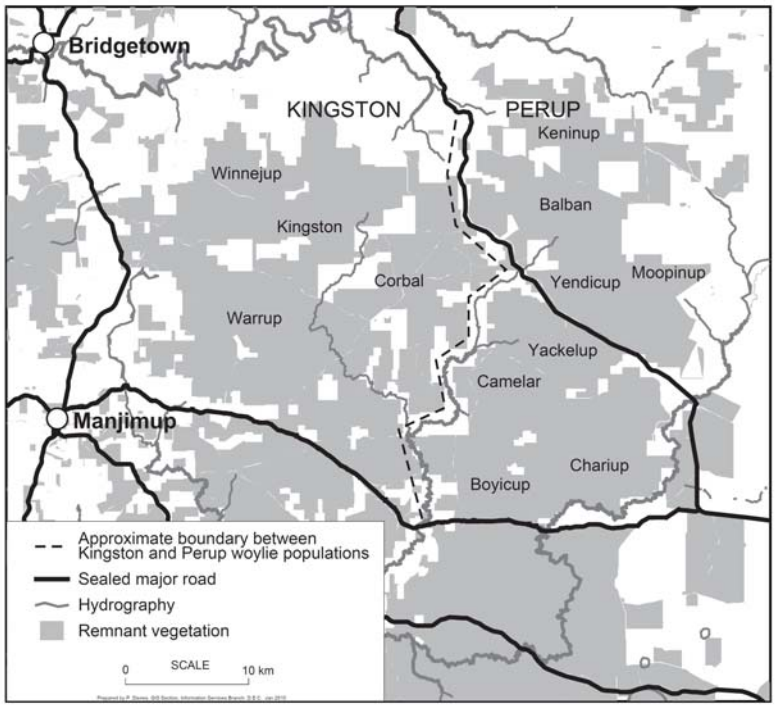

Figure 6.2 Woylie populations and sampling sites within the Upper Warren region.

6.3.2 DNA extraction and amplification

Complete genotypes at 12 microsatellite loci were determined for 231 adult woylies using methods described in Pacioni and Spencer (2010). In addition, a partial (~600bp) section of the tRNA Proline-end of the control region (or D-loop) was amplified by means of the polymerase chain reaction (PCR) using the primers H15999M and L16498M and reaction concentrations described by Fumagalli *et al.* (1997) on a subset of 152 samples (Table 6.1). Reaction conditions were slightly modified using a preliminary denaturation step at 95°C for 3 min, then 40 cycles of: 95°C for 45 sec, 53°C for 45 sec, 72°C for 90 sec. This was followed by a final extension step at 72°C for 5 minutes.

PCR products were sequenced using dye terminator cycle sequencing chemistry (3730xl sequencer; Applied Biosystems). The DNA sequences were compared to those in the Genbank database using the basic local alignment tool (http://www.ncbi.nlm.nih.gov/ BLAST/) to confirm that the correct product was amplified. Sequences were aligned using the progressive pair-wise alignment algorithm (Drummond et al. 2007) incorporated in Geneious Pro 3.8 (Biomatters) and then the alignment was manually checked.

6.3.3 Phylogenetic analysis

The software MEGA v.4 (Tamura et al. 2007) was used to reconstruct phylogeny history using the neighbour-joining (NJ) and minimum evolution (ME) tree-searching methods under the Maximum Composite Likelihood substitution model (Tamura et al. 2004) with complete deletion of sites with gaps or missing data, leaving 563 sites for analysis. The rate of variation among sites was estimated in PAUP 4.0 (Swofford 2005) and finally modelled with a gamma distribution (shape parameter = 0.6991). Northern bettong (*Bettongia tropica*) was used to root the tree due to its position as a sister taxon to *B. penicillata* and reliability estimated from 1000 bootstrap replications. Under the same setting, we used MEGA (Tamura et al. 2007) to compute the mean evolutionary distance between haplotypes. MrBayes (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) was used to perform a Bayesian analysis. Briefly, the analysis was conducted with flat priors, carrying out two runs of four chains each of 6,000,000 generations with sampling every 100 generations. PAUP 4.0 (Swofford 2005) was used to estimate parameters for 56 substitution models based on the neighbour joining tree of Jukes-Cantor distances and then Modeltest version 3.7 (Posada & Crandall 1998) was used to select an appropriate substitution model using the corrected Akaike information criterion (AIC) as suggested by Posada and Buckley (2004). Parameter estimates derived from Modeltest

were successively implemented in MrBayes analysis. We used the MrBayes 'convergence diagnostic' function to determine whether the two runs had converged (average standard deviation <0.2) and discarded the first 15,000 trees as a 'burn-in' to obtain a 50% consensus tree. Additionally, we verified the likelihood profile in TRACER version 1.1 (Rambaut & Drummond 2007) to ensure that the 'burn-in' was sufficient.

6.3.4 Microsatellites analysis

Genotypic data were initially manipulated using Microsoft Excel, and were checked for errors, and input files were created for other programs using the export function in GENALEX 6.2 (Peakall & Smouse 2006). Population structure was investigated between the sampling units using assignment tests to identify genetic structure and to assign individuals to their likely population of origin with STRUCTURE 2.2 (Pritchard et al. 2000). The analysis was repeated with and without the marker Y151 because of the surprising size range of this marker (Pacioni & Spencer 2010). STRUCTURE uses a Bayesian assignment approach to determine the most likely number of *inferred* populations (*K*), based on the observed genotypes, and determine the extent of the contribution from each inferred population to each animal's genotype. A putative population is a group of samples obtained from a discrete geographic area and an inferred population is a collection of samples that clustered together according to the assignment results. To determine the most likely number of populations, we analysed the posterior probability of the data given K (log Pr(X/K)) (Pritchard et al. 2000) and the second rate of change of the likelihood distribution, ΔK (Evanno et al. 2005). STRUCTURE assumes that the distribution of alleles conforms to Hardy-Weinberg equilibrium, that there is no linkage between markers (Pritchard et al. 2000) and allows analysis assuming that individuals originated from a population that had common ancestors (admixture) or alternatively that

they are from genetically independent populations. Tests assuming each scenario were carried out. Also, under the admixture model, the hypothesis that the allele frequencies were correlated or alternatively that they were not, have been tested. Lastly, the software allowed the inclusion in 'the prior probability' of the model the information about the putative populations. In case this information was wrong the analysis would overcome these 'miss assignments' if the genetic signal was strong. Tests with 'blind' and two different 'informed' prior settings were carried out as follows. A first analysis was performed assuming as putative populations each forest block in Upper Warren. Successively, the analysis was repeated with the dataset organised according to the preliminary results, to confirm migrant animals under a more restricted model. Each STRUCTURE result was based on 20 independent runs from one to 25 (K = 1-25) inferred populations, using a 'burn-in' period of 100,000 iterations followed by 10^6 iterations of a Markov Chain Monte Carlo. When the dataset was reorganised according to the preliminary results, K-values were limited to 10, with no changes in all the other conditions.

6.3.5 Genetic diversity

The quality of the data and early detection of null-alleles and allelic dropout was checked with MICRO-CHECKER (Van Oosterhout et al. 2004). Hardy-Weinberg equilibrium and linkage disequilibrium were tested with HW-QUICKCHECK (Kalinowski 2006) and GENEPOP 4.0 (Rousset 2008), respectively. Descriptive measures of population genetic diversity were all calculated using GENALEX 6.2 (Peakall & Smouse 2006) and included measures of observed (H_o) and expected heterozygosity (H_E), observed (N_A) and expected numbers of alleles (N_E) and average number of private alleles (PA). To further enable the comparison of the genetic variability among populations, we calculated the average allelic richness (N_{AR}) and average

private allelic richness (PA_R). These parameters were based on 28 diploid individuals using the rarefaction method implemented in HP-RARE (Kalinowski 2005), which compensates for differences in sample size producing unbiased estimates of allelic richness and then compared N_{AR} with the non-parametric Wilcoxon signed-rank test using SPSS v.15. The hierarchical population structure was further defined by calculating the estimator of genetic differentiation, F_{ST} in GENALEX 6.2 (Peakall & Smouse 2006) under the AMOVA framework using 1000 permutations to test significant difference from zero.

6.3.6 Gene flow

We estimated migration using both direct and indirect methods. The term migration is classically used to indicate displacement of an individual from one genetic population to another (Allendorf & Luikart 2007). However, in ecological studies it is more frequently referred to as a dispersal event and the term migration is reserved to indicate common movements of a species during different seasons or their life cycle (Allendorf & Luikart 2007). Here, the terms migration and dispersal are used indifferently.

6.3.6.1 Direct estimates

Direct dispersal estimates are usually based on ecological approaches (e.g. mark-recapture or radio tracking studies). A limitation of these methods is the difficulties to detect occasional or irregular dispersal events. Molecular ecology can provide a valid contribution in that it is possible to assign an individual to its most likely source population according to its genotype. Using the information generated by STRUCTURE under the admixture model with correlated allele frequencies and including in the prior the information of the putative populations, we

identified recent (F_0, F_1 or F_2 generation) migrants between wild populations and calculated N_m estimates weighting for the generation in which the dispersal event took place. That is, F_0 account for 1, F_1 for 0.5 and F_2 for 0.25. DNA of migrants was re-extracted and their profiles were re-genotyped to ensure that no genotyping errors occurred.

6.3.6.2 Indirect estimates

The effective number of migrants between any two inferred populations per generation (N_m) was estimated using F_{ST} values according to Peakall et al (1995), based on allele frequencies methods, using the relationship of N_m=(1/ F_{ST} -1)/4 (Slatkin 1985). We used F_{ST} to obtain indirect measures of gene flow (N_m) between populations because this estimate is based on historical rates of gene flow and it is considered a better estimator of migration rate than R_{ST}, for studies such as this involving low numbers of loci being scored (n<20; Gaggiotti et al. 1999). The limitations of this approach are mainly related to the assumptions implied that are rarely met in natural populations (Hardy-Weinberg equilibrium, populations of equal and constant size, constant and symmetric migration rates, neutral selection, no or negligible mutation, migration-drift equilibrium, same probability of reproduction for migrant and resident individuals). Moreover, this approach has been developed under the island model of migration (Slatkin 1985), which may not be always the real scenario.

An alternative approach is to estimate migration using the 'private allele' method (see Barton & Slatkin 1986) and we used GENEPOP 4.0 (Rousset 2008) for this purpose. This approach too assumes the island model of migration. Another constraining factor in the assumptions is that the migration has to be much higher than mutation rate because the model does not take into account mutation. Despite the limitations of these two methods, they are still widely used and can provide an indication of the general trend (e.g. low versus high migration rates) rather than the exact number of migrants (Allendorf & Luikart 2007).

Table 6.1 Summary of the samples collected in each sampling location, measures of microsatellite variability [mean (± SE)] and genetic contribution (given as a proportion) of each of the four inferred population clusters.

	1. Dryandra	2. Tutanning	3. Kingston	4. Perup
n(MS)[a]	28	32	69	102
n(mtDNA)[b]	8	13	48	83
N_A[c] *(SE)*	8.92 (± 0.92)	5.5 (± 0.61)	12 (± 1.33)	15 (± 1.81)
N_E[d] *(SE)*	5.76 (± 0.66)	3.23 (± 0.31)	5.91 (± 0.62)	7.55 (± 0.88)
N_{AR}[e] *(SD)*	8.92 (± 3.07)	5.4 (± 1.92)	10.05 (± 3.37)	12.03 (± 3.89)
H_E[f] *(SE)*	0.796 (± 0.03)	0.64 (± 0.05)	0.788 (± 0.04)	0.835 (± 0.03)
H_o[g] *(SE)*	0.731 (± 0.046)	0.645 (± 0.078)	0.706 (± 0.058)	0.746 (± 0.039)
PA[h] *(SE)*	0.92 (± 0.31)	0.67 (± 0.41)	1.17 (± 0.42)	2.42 (± 0.89)
PA_R[i] *(SD)*	1.42 (± 1.61)	0.86 (± 1.32)	1.47 (± 1.43)	1.99 (± 2.18)
Cluster 1	**0.998**	0.001	0.005	0.002
Cluster 2	0.001	**0.998**	0.001	0.001
Cluster 3	0.001	0.000	**0.987**	0.025
Cluster 4	0.001	0.001	0.008	**0.972**

[a] number of individuals genotyped at microsatellite loci.
[b] number of DNA sequences generated at the mitochondrial DNA control region.
[c] average number of alleles.
[d] average effective number of alleles.
[e] average allelic richness.
[f] expected heterozygosity.
[g] observed heterozygosity.
[h] average private alleles.
[i] average private allelic richness.

6.4 Results

Phylogenetic trees had similar topology with all the methods used. Nucleotide composition was highly biased towards thiamine (T: 40.3 C: 5.9 A: 26.7 G: 27.1) and the overall transition/transversion bias was *R* = 6.645. Fifteen haplotypes (GenBank accession number: HQ141321-HQ141335) were identified and their geographic distribution is shown in Fig 6.3. Average genetic distance of the 15 haplotypes was 0.02196 (SE= 0.0047).

STRUCTURE (Pritchard et al. 2000; Falush et al. 2003) identified the existence of four genetically distinct groups (i.e. K = 4). No difference in this result was obtained when marker Y151 was removed from the dataset. Two of these clusters, Dryandra and Tutanning, are consistent with their geographic separation, while Upper Warren was divided in two different populations: one that comprises the western blocks (from now on identified as Kingston) and the second includes the remaining blocks in the east (Perup. Fig. 6.2). All the analyses that included the geographic information in the prior, generated a mode at K=4 for both distribution: the posterior probability of the data given K (log Pr(X/K)) (Pritchard et al. 2000) and the second rate of change of the likelihood function, ΔK (Evanno et al. 2005). Interestingly, with the no-admixture model and no-correlated allele frequencies under the admixture model (and no geographic information in the prior), the distribution of ΔK showed a mode at K=3, with Dryandra and Perup grouped together while the log Pr(X/K) constantly showed higher values at K=4.

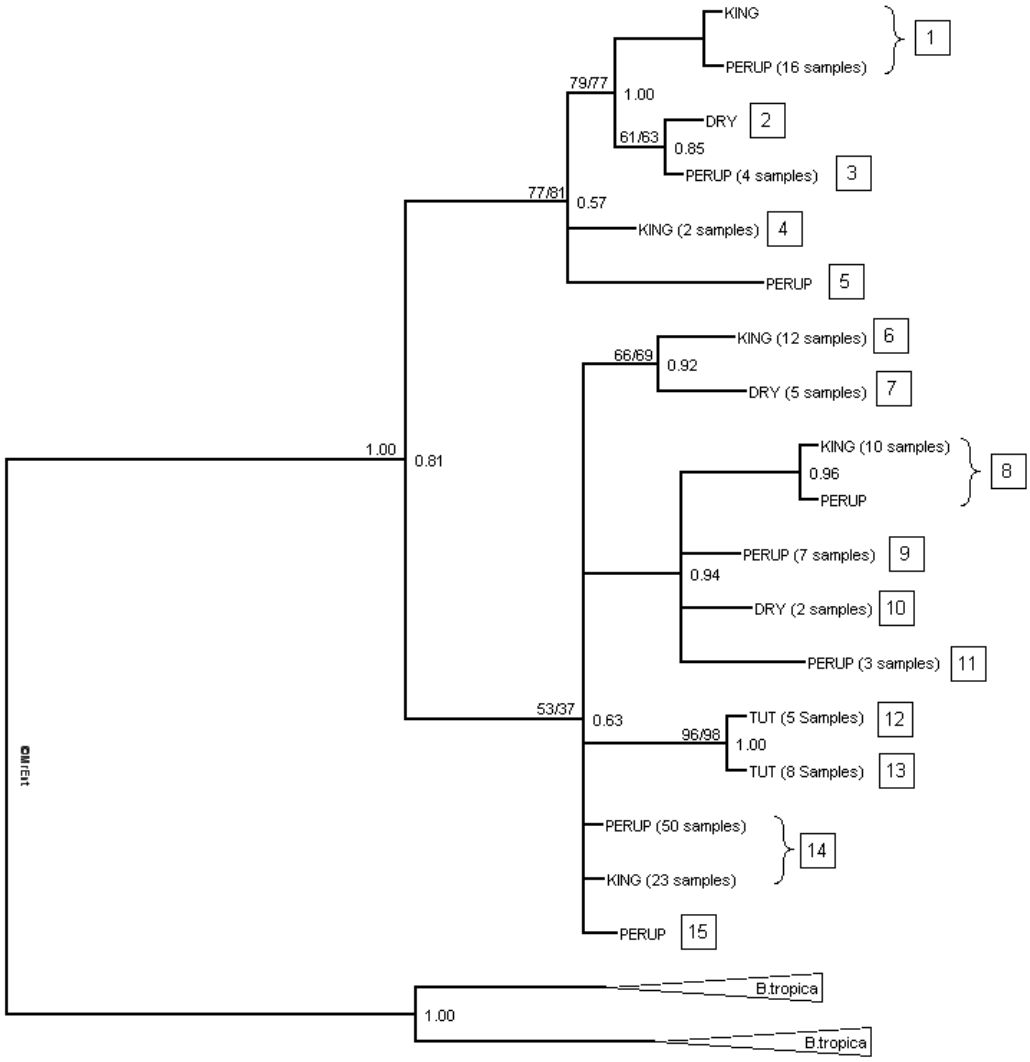

Figure 6.3 Phylogenetic tree.

More than 0.5 Bayesian posterior probabilities are reported internally to the nodes. If bootstrap support percentages were more than 50%, these were reported above the branches (NJ/ME). Numbers in squares represent the number of different haplotypes. If more than one sample from the same location had the same haplotype the total number of samples is indicated between brackets.

Several animals were identified as migrants between various populations. Table 6.2 reports details of these individuals based on the most conservative model. Running the admixture model without using any information about the putative populations, the number of animals, whose fraction of genotype derived from other populations, increases. Interestingly, another four animals appear to be related to Dryandra while being trapped in Upper Warren and two additional woylies for each side of Upper Warren appear to be migrants from the opposite side (data not shown).

Table 6.2 Details of woylie individuals indentified as migrants between populations under the admixture model with correlated allele frequencies and using geographic information.

ID	Sex	(%Miss)[a]	Into	From	Generation	P(As)[b]	P(Anc)[c]
07-366	M	0	Kingston	Perup	F2	0.069	0.929
07-370	M	0	Kingston	Dryandra	F2	0.01	0.988
07-381	F	0	Kingston	Perup	F2	0.546	0.434
07-341	F	0	Perup	Kingston	F1	0.04	0.951
07-389	M	0	Perup	Kingston	F0	0	1
07-585	M	0	Perup	Kingston	F0	0	1

[a] percentage of genetic data missing.
[b] probability to be assigned to the putative population.
[c] probability to have ancestors in the inferred population.

Of the 48 tests carried out to verify Hardy-Weinberg proportions, six were significant after Bonferroni correction. However, no consistent pattern was evident across populations, or loci, and consequently we judged that the results depended more on the single population rather than a problem with these particular loci.

All populations had expected levels of heterozygosity (H_E) of around 0.8 except for Tutanning with 0.64 (Table 6.1). Not surprisingly, Tutanning also had the lowest N_A 5.5 (± 0.61), N_E 3.23 (±

0.31), *PA* 0.67 (± 0.41) and PA_R 0.86 (± 1.32) (Table 6.1). Furthermore, N_{AR} was significantly lower at Tutanning than the other populations (P=0.002). Perup showed the highest N_{AR} when compared with the other populations (P<0.005). The analysis of molecular variance among populations was significantly different from zero (Table 6.3) with values ranging from 0.056 to 0.164. The population in Tutanning showed the highest level of differentiation when compared with the other populations sampled.

Table 6.3 Pairwise estimates of F_{ST}.

	Dryandra	Tutanning	Perup	Kingston
Dryandra		0.001	0.001	0.001
Tutanning	0.152		0.001	0.001
Perup	0.061	0.137		0.001
Kingston	0.089	0.164	0.056	

Probability values based on 1000 permutations are shown above diagonal.

Direct migration estimates (N_m) were 2.5 migrants from Kingston to Perup and 0.5 migrants from Perup to Kingston, while only one animal was constantly detected as F_2 migrant from Dryandra to Kingston (N_m=0.25). N_m estimated with the allele frequencies and private alleles approaches were very low and slightly different (Table 6.4). Given the previously mentioned limitations of these methods, we also show the relative rates of these measures (Table 6.4). To illustrate this we used as a unit the *rate* between Dryandra and Tutanning and weighted the other values. In this way, it was evident that with both methods the connection between Dryandra and Upper Warren was about twice as strong as the one between Dryandra and Tutanning and the connection between the two Upper Warren populations was around three times as strong.

Table 6.4 Number of migrants calculated with allele frequencies (first line) and private allele methods (second line).

	Dryandra	Tutanning	Kingston
Tutanning	1.39 (1)		
	0.54 (1)		
Kingston	2.53 (1.82)	1.28 (0.92)	
	0.98 (1.82)	0.75 (1.39)	
Perup	3.91 (2.8)	1.59 (1.14)	4.31 (3.09)
	1.11 (2.06)	0.85 (1.58)	1.4 (2.6)

Between parentheses are the relative migration values, using the value between Dryandra and Tutanning as unit (equal to 1).

6.5 Discussion

Four distinct indigenous woylie populations were identified in this study. Where previously the woylies in the Upper Warren were considered and managed as effectively the same population, it is now clearly and genetically evident that two exist – approximately separated by the Perup River and associated farmland, these are nominally called the 'Kingston' population in the west and 'Perup' population in the east (Fig. 6.2). Whether this distinction was historic or a more recent result of habitat fragmentation since settlement and agricultural development by Europeans (post 1829), remains to be unequivocally determined however, for reasons elaborated later, the latter is most likely.

Perhaps the most striking findings from this study relate to the differences between the Tutanning and other indigenous populations. Tutanning woylies were genetically distinct as demonstrated by the monophyletic clade resulting from the phylogenetic analyses of mtDNA haplotypes (Fig. 6.3) and STRUCTURE analyses. Consistent with the other analyses, N_m estimates provided supporting evidence for the lack of connectivity between Tutanning and the other indigenous populations, which is also likely to be a consequence of lack of

continuous suitable habitat. The genetic consequences of habitat fragmentation have been well demonstrated in other species (e.g. Bowyer et al. 2002; Banks et al. 2005). These together with other ecological and biological consequences of habitat fragmentation present particular challenges to the conservation and management of species and communities (Heinsohn et al. 2004; Wayne et al. 2006a; Fischer & Lindenmayer 2007), which are likely to be relevant to the woylie.

While distinctions between the four populations exist, it is also clear that there is genetic mixing, particularly between the two Upper Warren populations. The phylogenetic analyses also indicate that the mtDNA haplotypes in the Dryandra population are closely related to those in the Upper Warren. Therefore the three populations (Dryandra, Perup and Kingston) were historically connected. As such, these populations may be considered as being part of the same evolutionarily significant unit (Moritz 1999). In other non-vagile macropods there are examples of gene flow across similar spatial scales. Admixture of haplotypes in the yellow-footed rock wallaby (*Petrogale xanthopus*) from locations up to 70 km apart was considered evidence of historical connections between sites (Pope et al. 1996). Long distance migrations maintaining high gene flow across large areas have also been demonstrated in other small marsupials, such as the Western Pygmy Possum (*Cercartetus concinnus*) across South Australia and Western Australia (Pestell et al. 2008).

Significant gene flow between the two Upper Warren populations (Kingston and Perup) is also evident with remarkable concordance in the N_m estimates derived by three independent methods (direct estimates, allele frequencies and private alleles). Using the most conservative approach, between 2% and 3% of woylies from these populations were estimated as migrants. With due consideration of the limitations to make direct comparisons because of the differences in methodology, these values are similar to those observed in other macropods.

For example, based on assignment tests, 12% (n=17) of northern bettongs were identified as migrants (Pope et al. 2000) and 5% in the brush-tailed rock-wallaby (Piggott et al. 2006). A 6% dispersal rate in juvenile yellow-footed rock-wallaby has also been observed (Sharp 1997).

Contrary to expectation, the degree of genetic divergence among indigenous populations was not related to their geographic distances. Despite the close proximity between Tutanning and Dryandra (< 40 km), the former is notably divergent from the other indigenous populations. Additionally, the two large Upper Warren populations did not show further genetic substructure. The potential for large-scale woylie movements that might prevent substructuring is demonstrated by the evidence of gene flow across the Upper Warren (e.g. N_m) and experimental as well as field observations of individuals moving 3-9 km (Christensen 1980; Pacioni et al. Submitted). This is in contrast with the northern bettong where, in seemingly continuous habitat, populations as close as 12 km were genetically distinct (Pope et al. 2000). However, male boodies (burrowing bettong, *B. lesueur*) were capable of dispersing up to 6 km (Parsons et al. 2002) and substantial migration in the rufous bettong (*Aepyprymnus rufscens*) has been demonstrated, in spite of significant genetic divergence among populations within a 6.5 km radius (Pope et al. 2005).

It is extremely unlikely that the historically evident movements between Dryandra and Upper Warren are still naturally occurring given the extent of habitat fragmentation and patchiness of remnant woylie populations. However, one individual in Upper Warren was constantly assigned as having ancestors (within the last two generations) from Dryandra. This is possibly the result of a human-assisted movement. Woylie joeys commonly come into the care of humans and are then transported throughout southwest Western Australia. It is therefore plausible that one or a number of these animals (or their offspring) have been subsequently released into the wild and effectively translocated to another population. Furthermore, there

is no record of animals moving between Dryandra and Upper Warren despite intensive and regular trapping in these areas over the last 35 years. It is also possible that this seemingly spurious case could be an artefact of historical connectivity and that this individual may represent an 'echo' of past gene flow between these two populations.

Relatively high genetic variability in Dryandra, Kingston and Perup woylie populations were repeatedly demonstrated (e.g. H_E, N_A, N_E, N_{AR}, PA and PA_R). These populations are therefore likely to have reasonable potential medium-long term genetic viability. H_E in these populations (0.78-0.83) was slightly higher than other *Bettongia* (*B. tropica*; H_E: 0.65-0.75, Pope et al. 2000; *B. lesueur*; H_E: 0.68-0.7, Donaldson & Vercoe 2008) and some other potorines (*Potoroos longipes*; H_E: 0.556, Luikart et al. 1997; *P. gilbertii*; H_E: 0.457, Sinclair et al. 2002; but *Aepyprymnus rufescens*; H_E: 0.83, Pope et al. 2005).

The genetic variability at Tutanning was substantially less than the other indigenous populations (H_E = 0.63). This finding is likely to be a result of genetic drift, probably consequence of the small population size being sustained for an extended period (i.e. approximately 300 animals for at least the last 40 years). It also suggests that the long term viability of this population could be compromised and soon at risk of inbreeding depression. Sampson (1971) stated that unless management actions were taken to restore habitat quality and continuity in the reserve to facilitate an increase in woylie population size and connectivity, Tutanning would effectively function as an island population. Interestingly, the H_E, N_A and N_E values at Tutanning were very similar to the woylie population on St Peter Island, South Australia, which is of comparable size to Tutanning (our unpublished data). H_E (0.63) at Tutanning was also lower than the H_E in boodies on Dorre Island (H_E: 0.68, Donaldson & Vercoe 2008) in Shark Bay (Western Australia), an arid island of about twice the size of Tutanning.

Ensuring the persistence at relatively abundant levels of all four indigenous woylie populations remains fundamental to the long-term conservation of this species. Each one of these populations retains unique genetic material and each can be regarded as a discrete management unit. Genetic diversity within the indigenous populations was relatively high at the time of sampling, except for Tutanning. The continuation of the woylie population declines throughout the south-western Australia and elsewhere should be of significant concern. Both the extent of the declines and particularly the duration to which a recovery may be delayed will result in increasing likelihood of genetic loss and associated consequences.

The causes and nature of the divergence of the Tutanning population could be resolved by further studies including the use of more conserved genome regions (e.g. cyt b) and an analysis of historic material available at museums and elsewhere. Monitoring the Tutanning population for signs of inbreeding depression would also be recommended.

In terms of disease transmission risks, this study indicates that woylies, at least within Upper Warren, are able to directly carry and transmit disease across the whole area. Evidence of possibly recent animal movement from Dryandra to Upper Warren (regardless whether human-assisted or not) also raises the potential for disease transmission across greater distances.

Lastly, we recommend including regular genetic monitoring, e.g. every 3-6 generations (i.e. 6-12 years), in the management plan for the woylie. This would directly enhance conservation prospects for this species but also presents a significant and unique opportunity to improve our understanding of genetic dynamics during population declines that may be relevant to species conservation more broadly. The exceptional value of the woylie as a potential 'model' for developing our understanding is accentuated by the well-documented history of woylie

declines, translocations and past recovery during the 20th Century (Orell 2004), the depth and breadth of associated ecological and demographic baseline data available (particularly over the last 35 years, e.g. Burrows & Christensen 2002) and the foundations established by this study.

6.6 Acknowledgments

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7 Genetic consequences of the founder effect and limited carrying capacity on translocated populations using the critically endangered woylie (*Bettongia penicillata*) as a model.

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7.1 Abstract

Translocations are an important management option for conservation purposes. The establishment and the demographic growth of a translocated population is not sufficient to declare it a success, but adequate genetic representation of the source and long term viability

of the newly founded populations should also be considered. We reviewed the translocation history of the woylie (*Bettongia penicillata ogilbyi)* from a genetic perspective using mitochondrial DNA and nuclear (microsatellite) loci and showed that the recipient populations had reduced genetic variability, diverged from their source and the supplementation carried out in two island populations appeared to have failed. The genetic diversity and the degree of divergence of the translocated populations were a function of the number of founders, time since establishment and population sizes. We envisage that the only population (Karakamia Sanctuary) that adequately represented the source population (Dryandra) will require ongoing and active management in order to preserve its remaining genetic diversity. We further discuss the conservation implications that our results have for the species, outlining general recommendations for the management of present and future translocations and discussing the appropriate sampling design for the establishment of new populations or captive breeding program. This research improved our understanding of translocation biology and its findings are directly applicable to other macropods, since woylies were shown to be an optimal model for species with similar ecology.

Keywords Bettong, macropod, translocation, supplementation, microsatellites, mtDNA

7.2 Introduction

Translocations are an important management strategy for biodiversity conservation (Griffith et al. 1989; Wolf et al. 1996; IUCN 1998; Seddon et al. 2007). However the effectiveness and value of translocation programs can be compromised by a reduction of genetic diversity, which can occur even in successfully established translocated populations (Stockwell et al. 1996;

Goossens et al. 2002). A reduced genetic variability can limit the evolutionary potential (Frankham 1996; Frankham et al. 1999) and reduce the fitness of populations by decreasing fecundity and survival rates (Ralls et al. 1988; O'Grady et al. 2006) and increasing susceptibility to diseases (e.g. Acevedo-Whitehouse et al. 2003; Spielman et al. 2004; Siddle et al. 2007; Charpentier et al. 2008). Consequently, genetic studies have been recommended to assess the appropriateness of translocations as an effective conservation option (Stockwell et al. 1996; IUCN 1998; Moritz 1999). Genetics in combination with demographic monitoring should be used for the assessment of translocation success (Goossens et al. 2002). Investigations of the genetic profile of founders and genetic variability of the translocated populations could increase the likelihood of success, help to predict the long-term viability of the new populations, and quantify the success of subsequent supplementations (the addition of individuals to an existing population) by providing information on the reproductive success of recently translocated individuals (Goossens et al. 2002 and within).

In Australia, translocations have been considered an important tool in attempting the re-establishment of a species in its previous range. For example, within the Western Shield program, 88 translocations (25 species) were carried out involving three states (Western Australia, South Australia and New South Wales) between April 1996 and September 2002 (Mawson 2004). However, success has been hindered by an inefficient control of threats such as feral predators (Fischer & Lindenmayer 2000).

Several studies reviewed macropod translocations in Australia in the attempt to identify the most common problems and establish best practices (e.g. Short et al. 1992; Mawson 2004; Finlayson et al. 2010). For example, 52 translocations (59.1%) carried out under the Western Shield program involved members of this superfamily (Mawson 2004).

The woylie (brush-tailed bettong, *Bettongia penicillata ogilbyi)* has a chequered conservation history, but it has been suggested as an optimal model for translocations due to their high reproductive rates, abundant source populations (which have sharply declined in the last few years), their adaptability to a variety of climatic conditions and the considerable knowledge gained in the last few decades about the ecology and physiology of this species (e.g. Mawson 2004; Finlayson et al. 2010). Additionally, the woylie is one of the most extensively translocated native species and its translocation history, beginning in the early 1970s, offers an excellent opportunity to empirically review the genetic outcomes of translocations as a conservation strategy in a variety of scenarios. In fact, several translocated populations were established in the last few decades: from small populations on isolated islands through to large successful reintroductions on mainland sites; with founders either directly sourced from the wild or through breeding programs (Mawson 2004).

By the 1970s the woylie had declined dramatically, was limited to Western Australia and listed as *Endangered*. The conservation strategies in place between the 1990 and 1995 effectively promoted the recover of the species (Start et al. 1998). Nevertheless, the woylie is now listed as *Critically Endangered*, having declined by 80% between 2001 and 2006, (Wayne et al. 2009; Groom 2010). This alarming and recent decline has resulted in a timely review of the management of this species.

Four genetically distinct naturally occurring woylie populations were identified (Pacioni et al. 2011): two in the Wheatbelt (*Dryandra* Forest and *Tutanning* Nature Reserve (Fig. 7.1)) and another two in the Jarrah forest of the Upper Warren region (*Kingston* and *Perup*, (Fig. 7.1)). With the exception of a preliminary genetic study of the translocated populations in South Australia, which indicated a low level of genetic diversity – 80% band sharing of VNTR (Variable Number Tandem Repeat) – (Start & Armstrong 1994; Start et al. 1998), no thorough genetic

studies have been carried out to date on any of the translocated populations, despite the recommendations in the woylie recovery plan (Start et al. 1995).

We investigated the genetic profiles of the translocated populations using both mitochondrial DNA (mtDNA) and nuclear (microsatellite) markers and used this information to evaluate the success of the translocations, estimate levels of genetic diversity; establish the source population where it was unknown; and investigate factors that could have influenced the outcome.

7.3 Methods

7.3.1 Sample collection

Genetic data from Pacioni et al (2011) were used in this study for the four naturally occurring woylie populations (*Dryandra* Forest and *Tutanning* Nature Reserve in the Wheatbelt region and *Kingston* and *Perup* in the Upper Warren region (Fig. 7.1. Table 7.1)). Furthermore, in 2004 woylies were trapped using standard techniques from two translocated populations in Western Australia: *Batalling* State Forest (Fig. 7.1) and *Karakamia* Wildlife Sanctuary (Fig. 7.1). Founders of these populations were sourced from *Perup* and *Dryandra*, respectively. In 2006, samples were obtained from three additional translocated populations established on three islands in South Australia (SA) free from feral predators: St Peter Island (StPI), Wedge Island (WEDI) and Venus Bay Island (VBIA). Woylies translocated to the South Australian islands were sourced from a breeding program that was established with only three individuals (two females and one male from Perth Zoo) of unclear and undocumented Western Australian origin (Delroy et al. 1986). Two attempts were made to increase the genetic variability of the

population on WEDI, one in 1994 (releasing ten males) and in 1995 (releasing 15 males) but both failed with most animals found dead or not re-trapped in subsequent monitoring (Start & Armstrong 1994; Van Weenen 1996). In 1996, 15 woylies (ten males, five females) from *Dryandra* were released on StPI, but only six (four males, two females) were found in good condition after only two months following the release (Van Weenen 1996). Consequently the extent of their genetic contribution remains unknown. Further details about the populations included in this study, such as year of establishment, total number of animals released and population size, are summarised in Table 7.2.

7.3.2 DNA extraction and amplification

A modified high-salt method (Miller et al. 1988) was used for the DNA extractions and PCR amplification of 12 microsatellite loci were carried out following Pacioni and Spencer (2010). A partial (~600bp) section of the tRNA Proline-end of the control region (or D-loop) was amplified using the primers H15999M and L16498M (Fumagalli et al. 1997). Reaction conditions were as described by Pacioni et al (2011).

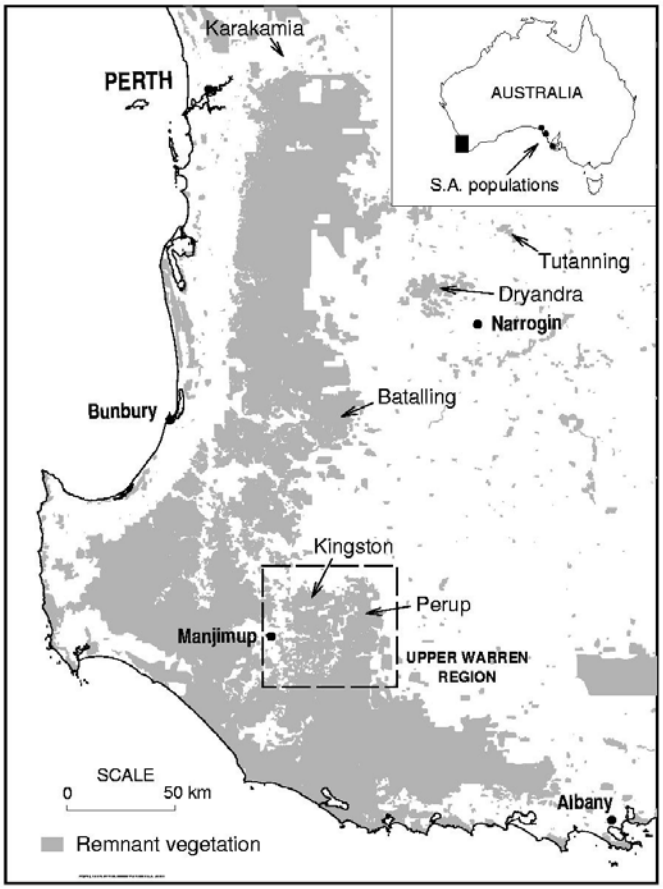

Figure 7.1 Geographical location of sampled woylie populations (modified with permission from Pacioni et al. 2011).

7.3.3 Sequence data analysis

The software MEGA v.4 (Tamura et al. 2007) was used to reconstruct phylogenetic history using the neighbour-joining (NJ) and minimum evolution (ME) tree-searching methods under the Maximum Composite Likelihood substitution model (Tamura et al. 2004) with complete deletion of sites with gaps or missing data, leaving 563 sites for analysis. The rate of variation among sites was estimated in PAUP 4.0 (Swofford 2005) and finally modelled with a gamma distribution (shape parameter = 0.6991). The Northern bettong (*Bettongia tropica*) was used to root the tree due to its position as a sister taxon to the woylie and reliability estimated from

1000 bootstrap replications. MrBayes (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) was used to perform a Bayesian phylogenetic analysis. Briefly, the analysis was conducted with "flat prior probabilities", carrying out two runs of four chains each of $6x10^6$ generations with sampling every 100 generations. PAUP 4.0 (Swofford 2005) was used to estimate parameters for 56 substitution models based on the neighbour joining tree of Jukes-Cantor distances and then Modeltest version 3.7 (Posada & Crandall 1998) was used to select an appropriate substitution model using the corrected Akaike information criterion (AIC) as suggested by Posada and Buckley (2004). Parameter estimates derived from Modeltest were successively implemented in the MrBayes analysis. We used the "convergence diagnostic" function of MrBayes to determine whether the two runs had converged (average standard deviation <0.2) and discarded the first 15,000 trees as a "burn-in" to obtain a 50% consensus tree. Additionally, we verified the likelihood profile in TRACER version 1.1 (Rambaut & Drummond 2007) to ensure that the "burn-in" was sufficient.

7.3.4 Microsatellites analysis

We carried out an assignment test using STRUCTURE 2.2 (Pritchard et al. 2000). STRUCTURE uses a Bayesian assignment approach to determine, based on the observed genotypes, the most likely number of inferred populations (*K*) and the extent of the contribution from each inferred population to each animal's genotype. Analysis of the data was repeated with both, the admixture and no admixture models (Pritchard et al. 2000). In addition, the hypothesis that the allele frequencies were or were not correlated, have been tested under the admixture model. We also tested whether adding information on the geographic location into the "prior probability" would alter the results. Using these settings, potential "mis-assignments" can be overcome by a strong genetic signal. To determine the most likely number of populations, we

analysed the posterior probability of the data given K (log Pr(X/K)) (Pritchard et al. 2000) and the second rate of change of the likelihood distribution, ΔK (Evanno et al. 2005). Each set of STRUCTURE results were based on 20 independent runs from one to 25 inferred populations (*K* = 1-25), using a "burn-in" period of 100,000 iterations followed by 10^6 iterations of a Markov Chain MonteCarlo. Population divergence was also estimated by calculating genetic differentiation, F_{ST} in GENALEX 6.2 (Peakall & Smouse 2006) under the AMOVA (analysis of molecular variance) framework using 1000 permutations to test significant difference from zero.

Descriptive measures of population genetic diversity were all calculated using GENALEX 6.2 (Peakall & Smouse 2006) and included estimates of genetic diversity within populations: observed (H_o) and expected heterozygosity (H_E); average of observed (N_A) and expected number of alleles (N_E); and average number of private alleles (*PA*). In order to further enable the comparison of the genetic variability among populations, we calculated the average allelic richness (N_{AR}) and average private allelic richness (PA_R) based on 14 diploid individuals using the rarefaction method implemented in HP-RARE (Kalinowski 2005), which compensated for differences in sample size producing unbiased estimates of allelic richness. We then compared N_{AR} of each locus with the non-parametric Wilcoxon signed-rank test in SPSS v.15.

By extension from Frankham (1998) the effective inbreeding coefficient, F_e, between translocated and source populations was calculated as:

$$F_e = 1\text{-}H_{TR}/H_S$$

Where H_{TR} and H_S are translocated and source populations, respectively.

Genetic evidence of population bottlenecks was investigated by testing for an excess in heterozygosity (Cornuet & Luikart 1996) and mode-shift (Luikart & Cornuet 1998), using the program BOTTLENECK (Piry et al. 1999). Due to the relatively small number of loci analysed (*n* = 12), a Wilcoxon sign-rank test was estimated, as recommended by Piry et al (1999). A mixed model of microsatellite mutation was assumed, with single step mutations accounting for 95% of all mutation events, and a variance among multiple steps of 12, as suggested by Piry et al (1999).

7.4 Results

7.4.1 Sequence data analysis

Phylogenetic trees showed identical topologies regardless of the method used to carry out the analysis, therefore only Bayesian post-probability values are presented (Fig. 7.2). In addition to the 15 haplotypes that had been already identified in the naturally occurring populations (Pacioni et al. 2011), two new haplotypes were identified: one in *Karakamia* and one in all three South Australian islands (GenBank accession number: HQ141336-HQ141337). *Karakamia* and the South Australian island haplotypes were either shared with, or closely related to, haplotypes found in *Dryandra*, which is the known source population of *Karakamia* (Fig 7.2). As expected, *Batalling* shared its two haplotypes with *Perup*, being its source population.

7.4.2 Microsatellites analysis

Inspecting the posterior probability of the data given K (log Pr(X/K)) (Pritchard et al. 2000) produced by STRUCTURE, the most probable number of inferred populations (*K*) was six regardless of the different priors and models used except for the no admixture model without geographic information, in which case it was five. In the analyses that did not include the geographic information in the prior, the second rate of change of the likelihood distribution, ΔK (Evanno et al. 2005), showed the highest peak at *K*=2 (where one of the cluster included all Western Australian populations and the other all South Australian populations) or *K*=6. The analyses that did include the geographic information in the prior generated the highest ΔK with *K*=6. Therefore, we concluded that *K*=6 was the most probable number of inferred populations. From now on, we refer to these groups as "genetic clusters", as "inferred populations" would not correspond to a biologically meaningful definition since some of these clusters grouped together populations that are geographically distinct (i.e. island populations or fenced area). Because there were only marginal differences in the proportion of membership of the sampling locations in each of the six genetic clusters among the different STRUCTURE runs, we report only the results for the run with the highest log-likelihood under the admixture model with correlated allele frequencies and geographic information included in the prior (Table 7.2). The assignment test correctly detected the known source population of *Karakamia*, which clustered together with *Dryandra*. *Batalling* and the three South Australian islands clustered in two distinct groups (Table 7.2). Nevertheless, the Bayesian assignment identified the common origin of the South Australian populations, which clustered together (despite significant F_{ST} values) and, in the analyses conducted with no geographic information in the prior, STRUCTURE correctly identified the link between *Batalling* and *Perup*. In fact, around 9% of *Perup* genetic profiles were assigned to *Batalling*.

The analysis of molecular variance among populations was significantly different from zero (Table 7.3, p-values above diagonal) with values ranging from 0.037 to 0.271. The F_{ST} value of the pair *Dryandra-Karakamia* was relatively small (0.046) and *Perup-Batalling* was moderate (0.065). Pairwise comparisons between the South Australian islands and *Dryandra* (source population based on mtDNA results) had higher F_{ST} values (0.164-0.231) with VBIA being the most differentiated. VBIA was also clearly different from the other two islands (F_{ST} = 0.136 WEDI and F_{ST} = 0.158 StPI), while these latter two were quite similar to each other (F_{ST} = 0.037).

All loci were polymorphic in all populations except locus Y112, which was fixed in the Venus Bay island population. N_{AR} was significantly lower ($p<0.05$) in all translocated populations when compared with their respective source populations. Not surprisingly, all the other genetic diversity measures (N_A, N_E, H_E, PA and PA_R) showed a similar trend (Table 7.1). Moreover, N_{AR} of StPI was significantly higher than N_{AR} in WEDI (p=0.023) and VBIA (p=0.008). The effective inbreeding coefficients (F_e) were very high for the island populations (0.208-0.469; Table 7.1) while it was relatively small for *Karakamia* (0.065) and intermediate for *Batalling* (0.137).

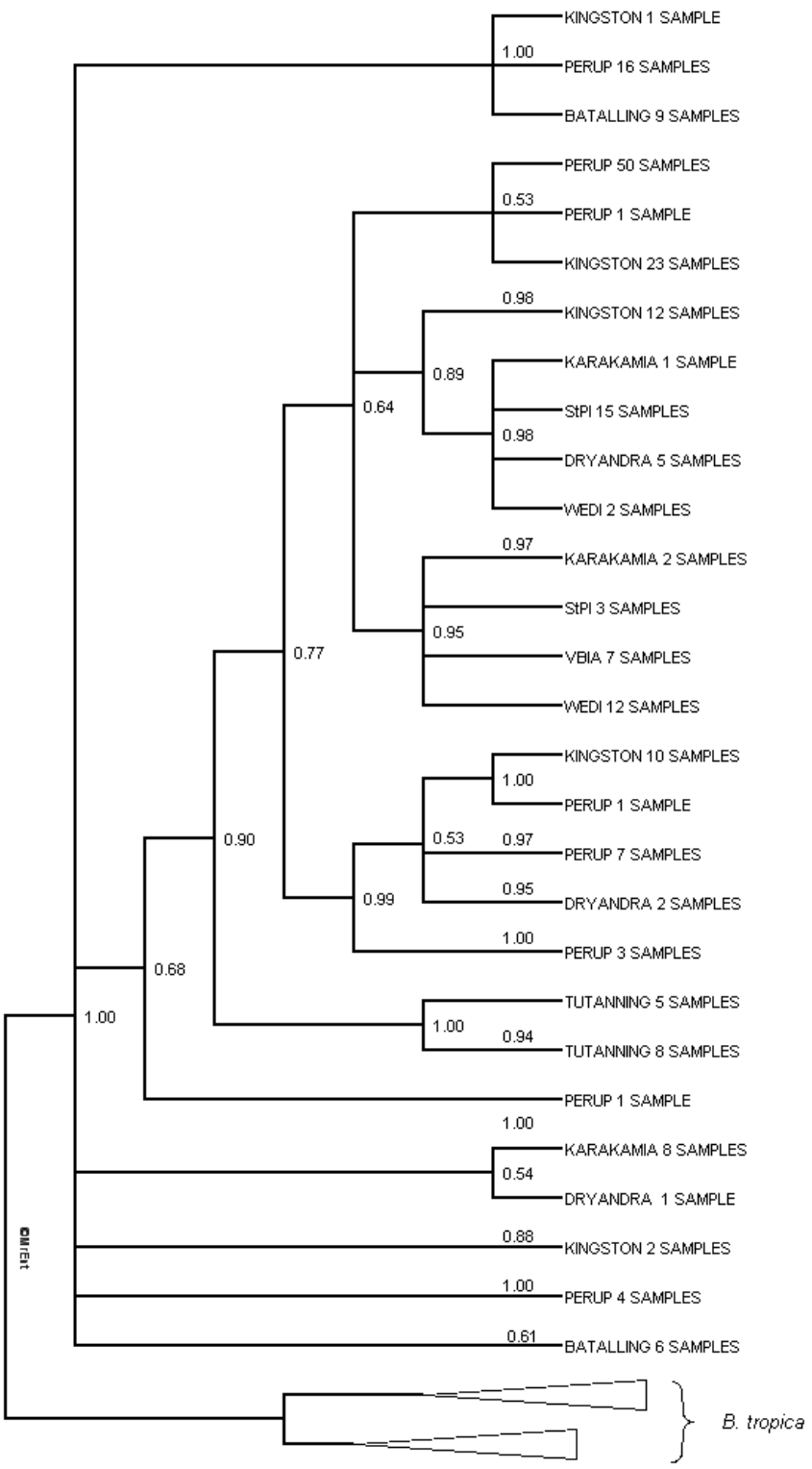

Figure 7.2 Bayesian tree based on the analysis of the partial D-loop (~600 bp).

More than 0.5 Bayesian posterior probabilities are reported.

Table 7.1 Summary of the samples collected during the study at each of the field sites (sampling locations) and measures of microsatellite variability

Sampled locality	State	n	N_A *(SE)*	N_E *(SE)*	N_{AR} *(SD)*	H_E *(SE)*	H_o *(SE)*	*PA (SE)*	PA_R *(SD)*	F_e
Dryandra	WA	28	8.92 (± 0.92)	5.76 (± 0.66)	7.83 (± 2.34)	0.796 (± 0.03)	0.731 (± 0.05)	0.25 (± 0.18)	0.6 (± 0.69)	NA
Karakamia	WA	29	7.5 (± 0.82)	4.87 (± 0.66)	6.67 (± 2.34)	0.745 (± 0.04)	0.661 (± 0.07)	0.17 (± 0.11)	0.26 (± 0.5)	0.06
Tutanning	WA	32	5.5 (± 0.61)	3.23 (± 0.31)	4.8 (± 1.49)	0.64 (± 0.05)	0.645 (± 0.08)	0.58 (± 0.34)	0.71 (± 0.9)	NA
Kingston	WA	69	12.08 (± 1.36)	5.93 (± 0.62)	8.23 (± 2.47)	0.788 (± 0.04)	0.706 (± 0.06)	1.08 (± 0.4)	1.23 (± 1.07)	NA
Perup	WA	102	15 (± 1.81)	7.6 (± 0.88)	9.72 (± 2.65)	0.836 (± 0.03)	0.746 (± 0.04)	1.67 (± 0.66)	1.03 (± 1.13)	NA
Batalling	WA	35	7.25 (± 0.57)	4.14 (± 0.43)	6.39 (± 1.58)	0.721 (± 0.04)	0.717 (± 0.05)	0.17 (± 0.11)	0.34 (± 0.33)	0.14
St Peter Island	SA	30	4.92 (± 0.54)	3.02 (± 0.27)	4.37 (± 1.21)	0.631 (± 0.04)	0.62 (± 0.05)	0.08 (± 0.08)	0.07 (± 0.2)	0.21
Wedge Island	SA	32	4.17 (± 0.3)	2.83 (± 0.22)	3.87 (± 0.95)	0.602 (± 0.06)	0.55 (± 0.06)	0 (± 0)	0.03 (± 0.1)	0.24
Venus Bay Island A	SA	14	2.67 (± 0.28)	2.1 (± 0.3)	2.67 (± 0.94)	0.423 (± 0.07)	0.455 (± 0.08)	0 (± 0)	0.03 (± 0.09)	0.47

n=number of individuals genotyped at microsatellite loci. N_A=average number of alleles. N_E=average effective number of alleles. N_{AR}=average allelic richness. H_E=expected heterozygosity. H_o=observed heterozygosity. *PA*=average private alleles. PA_R=average private allelic richness. *SE*=standard error. *SD*=standard deviation. F_e=effective inbreeding coefficient (Frankham 1998). WA=Western Australia. SA=South Australia.

Table 7.2 Population details and genetic contribution (given as a proportion) of each population to the six genetic clusters identified with STRUCTURE (Pritchard et al. 2000)

Sampled locality	State	Area (ha)	Type	Year	TNR	N_C 2001[a]	N_C 2006[a]	Genetic contribution to the genetic clusters					
								1	2	3	4	5	6
1. Dryandra	WA	12,192	N	-	NA	6,000	400-500	**1.00**	0	0	0	0	0
Karakamia	WA	275	T(*Dryandra*)	1994[b]	31[b]	500	500	**1.00**	0	0	0	0	0
2. Tutanning	WA	2,369	N	-	NA	300	300	0	**1.00**	0	0	0	0
3. Kingston	WA	25,000[c]	N	-	NA	20,000[d]	1,000[d]	0	0	**0.99**	0.01	0	0
4. Perup	WA	60,000[c]	N	-	NA	20,000[d]	1,000[d]	0	0	0.03	**0.97**	0	0
5. Batalling	WA	3,617[c]	T(*Perup*)	1983[e]	52[e]	3,000	400-500	0	0	0	0	**1.00**	0
6. South Australia													
St Peter Island	SA	3,493	T(Unknown)	1989[f]	113[fg]	2,000-3,500	2,000-3,500	0.01	0	0.01	0.01	0.01	**0.97**
Wedge Island	SA	947	T(Unknown)	1983[h]	11[gh]	1,500-3,000	1,500-3,000	0	0	0	0	0	**0.99**
Venus Bay Island A	SA	15	T(Unknown)	1980[h]	7[h]	30	30	0	0	0	0	0	**0.99**

The number in the first column indicates genetic cluster. Type:N=Naturally occurring; T=translocated, between brackets the source population. Year=Year of establishment. TNR=total number released. N_C=population census size. WA=Western Australia. SA=South Australia. NA=not applicable.

[a] (Groom 2010)

[b] J. Williams personal communication

[c] Approximate extent of occurrence, forest habitat is contiguous across a much larger area (i.e. not isolated and as discretely defined as wheatbelt and island populations.

[d] *Kingston* and *Perup* combined

[e] (Start et al. 1995)

[f] (Nelson et al. 1992)

[g] Additional releases (*Dryandra* stock) were carried out in 1996 (Van Weenen 1996).

[h] (Delroy et al. 1986)

None of the populations had a significant result from the one tail Wilcoxon test for heterozygosity excess. However, the power of these statistics was probably reduced by the length of time since establishment and the rapid growth of the new populations (Nelson et al. 1992; Orell 2004), which most likely caused a heterozygosity deficit rather than heterozygosity excess (Cornuet & Luikart 1996; Busch et al. 2007; Smith & Hughes 2008; Pacioni et al. Submitted).

Only VBIA showed signs of genetic bottleneck with the mode-shift test. Genetic drift is likely to be responsable for loss of rare alleles in this population (VBIA is the smallest of the three South Australian islands, with a census population size of 20-30 woylies).

Table 7.3 Pairwise population F_{ST} Values

	Dryandra	Perup	Batalling	Karakamia	St Peter Island	Venus Bay Island A	Wedge Island
Dryandra		0.001	0.001	0.001	0.001	0.001	0.001
Perup	0.061		0.001	0.001	0.001	0.001	0.001
Batalling	0.111	0.065		0.001	0.001	0.001	0.001
Karakamia	0.046	0.096	0.130		0.001	0.001	0.001
St Peter Island	0.164	0.153	0.205	0.206		0.001	0.001
Venus Bay Island A	0.231	0.222	0.271	0.264	0.158		0.001
Wedge Island	0.183	0.174	0.227	0.217	0.037	0.136	

F_{ST} Values below diagonal. Probability values based on 1000 permutations are shown above diagonal. *Tutanning* and *Kingston* were not included because these populations were not used as source population for any translocations.

7.5 Discussion

7.5.1 Genetic diversity of the translocated populations

All translocated populations had a significant reduction of genetic variability at microsatellite loci and a lower number of mtDNA haplotypes compared to the source populations. The loss of genetic diversity is the main genetic concern associated with translocated populations (Stockwell et al. 1996; Goossens et al. 2002) because, by definition, they go through a bottleneck and usually have a small population size (Frankham 1995a; Cornuet & Luikart 1996; Frankham 1996; Frankham et al. 1999; Frankham 2008). Additionally, the relatively short woylie generation time (less than 3 years, Groom 2010; 2.43 years, Pacioni and Wayne unpublished data) exacerbates this phenomenon. A degree of genetic diversity loss is unavoidable and efforts should be directed to limiting this loss to an acceptable level.

Despite the lower genetic variability, populations at *Batalling* (F_e=0.14) and *Karakamia* (F_e=0.06) were still a valuable representation of woylie genetic diversity. Expected heterozygosities (H_E) in these populations (0.721-0.745) were similar to what found in other *Bettongia* (*B. tropica;* H_E: 0.65-0.75, Pope et al. 2000; *B. lesueur;* H_E: 0.68-0.7, Donaldson & Vercoe 2008) and slightly higher than in other potorines (*Potoroos longipes;* H_E: 0.556, Luikart et al. 1997; *P. gilbertii;* H_E: 0.457, Sinclair et al. 2002; but *Aepyprymnus rufescens;* H_E: 0.83, Pope et al. 2005). These levels of H_E have been defined as "substantial" for macropod species (Pope et al. 2000) and, therefore, they can be considered acceptable.

It should be noted that the *Karakamia* population was established only ten years ago. Assuming that the founders' genetic diversity was similar among *Batalling* and *Karakamia*, it would appear that the smaller number of individuals released at establishment and the relatively small carrying capacity of the sanctuary (approximately 500 individuals) played a

significant role in the current levels of genetic diversity. The limited population size at *Karakamia* is expected to manifest the probability of genetic drift over time and close genetic monitoring is therefore warranted.

The *Batalling* and StPI populations were established almost at the same time and both reached similar population sizes (~3,000) with the only difference between these populations being the number founders. We argue that the small number of founders (3) of the breeding program used to establish the South Australian populations is responsible of the lower genetic diversity on the island. Overall, genetic diversity of the South Australian populations was compromised as shown by the high values of F_e (0.21-0.47) and other genetic variability parameters suggesting that active management is required in order to maintain the conservation value of these populations.

The supplementation goals of the South Australian populations were not achieved, given that the translocated populations had a reduced genetic diversity and had diverged from the source population. Although the StPI population had a slightly higher genetic diversity compared to the other two islands ($p<0.05$), it is difficult to discriminate whether this is an effect of the supplementation carried out in 1996 or an outcome of the larger size of the population. The latter explanation is in our opinion more likely.

7.5.2 Identification of source populations

The phylogenetic analysis linked the translocated populations to the known source populations (when available). We suggest that the relationship of the South Australian island haplotypes with *Dryandra* is evidence of the latter being the source of the founders of the breeding

program (at least for the females), from which the South Australian island populations were established. Reports that the South Australian captive colony was founded by only two females (Delroy et al. 1986) are also supported by this study, which identified no more than two haplotypes in the South Australian translocated populations. Furthermore, the analysis also provides evidence that females released during the supplementation exercises in 1996 did not contribute to the (mtDNA) genetic diversity of these populations. While it remains possible that their haplotypes were not different from those already present or that low frequency haplotypes were not sampled, it is clear that no effective genetic augmentation was achieved.

7.5.3 Implication for species management and conservation

This study suggests that a newly established population will be a replicate of the source population only for a few generations, even with ~50 founders and a population size of ~3,000 individuals. The degree of differentiation after only 20 years between the translocated populations at *Batalling* and three South Australian islands and their respective source populations (7-10 generations) further proves the vulnerability of translocated populations to the founder effect and the genetic drift (consequent to their population size). These populations not only had significant pairwise F_{ST} values, but also clustered independently from the respective source population in the STRUCTURE analysis. These results, consequently, highlight the limitations of translocation exercises that aim to replicate the genetic profile of the source population (i.e. insurance populations). In fact, can be only achieved as a short-term conservation measures. *Karakamia* was the only woylie population that adequately represented its source population (i.e. it did not cluster separately), but it is anticipated that this will not be sustained for many more generations. It is highly likely that the genetic profile of the *Karakamia* population will diverge from that of *Dryandra* unless active management is

undertaken. Similar differentiation was found to some extent in other Australian marsupials. For example, translocated populations of the brindled nailtail wallabies (*Onychogalea fraenata*) and the western barred bandicoot (*Perameles bougainville*) were found to have diverged from their source populations within ten years using Wright's F-statistics or its analogues (Sigg 2006; Smith & Hughes 2008). However, none of the western barred bandicoot populations were identified as separate clusters by the Bayesian assignment analysis (Smith & Hughes 2008) as in some of the woylie populations in this study.

Overall, translocated woylie populations are less genetically diverse than their founding counterparts. However, StPI and WEDI have been demographically stable in the last few decades (Groom 2010) and their geographic isolation represents a protection from factors that are difficult to control on the mainland (e.g. feral predators and diseases). Therefore, we recommend that these populations be managed as related subunits to overcome genetic problems associated with limited population sizes (e.g. through supplementations). Several studies suggested that a constant addition of “new” genetic material is a proper way to augment the genetic variability and reduce the divergence of a population from its source (e.g. Spielman & Frankham 1992; Sigg 2006; Smith & Hughes 2008). Despite the apparent failure of the supplementation attempt in 1996, we believe that this management option should not be abandoned. Careful consideration of factors that may affect the supplementation success, such as seasons or gender-specific survival differences, should be considered. In woylies survival of females after dispersal is higher than males (Christensen 1980), consequently, releasing small numbers of females would appear to be the best option when attempting further supplementations. Population modelling could provide valuable information (Allendorf & Luikart 2007; Seddon et al. 2007) and experimental trials should be carried out in order to optimise the supplementation methodology. Venus Bay island A would appear to be the optimum site for such trials due to logistics (proximity to mainland) and the relatively limited

conservation value of this population (carrying capacity of 25-30). For these experiments, sourcing woylies from rehabilitation centres could represent a cheaper and logistically easier solution compare to using wild caught individuals. Concurrently, precaution should be taken to minimise risks of disease transmission (e.g. adequate quarantine of translocated animals).

Tutanning, *Dryandra*, *Perup*, *Kingston, Karakamia* and *Batalling* can be considered as populations with an acceptable genetic diversity (Pope et al. 2000). If the consequences of recent declines become critical, admixture of individuals from naturally occurring populations may be acceptable (Moritz 1999) since these are within the same evolutionarily significant unit (ESU) (Pacioni et al. 2011; this study). On the other hand, admixture between *Tutanning* and any other populations should generally be avoided until future work confirm the evolutionary relationship of this population with the other Western Australia populations (Moritz 1999; Pacioni et al. 2011).

Limited population size appears to have disadvantaged the *Karakamia* and VBIA populations by increasing their susceptibility to genetic drift, while the limited number of founders was the main problem of the populations on the larger South Australian islands. The number of founders and carrying capacity appear to be important components that influence the final outcome and the genetic viability of the translocated populations in this and other species (e.g. Griffith et al. 1989; Wolf et al. 1996; Houlden et al. 1999; Fischer & Lindenmayer 2000; Larson et al. 2002; Maudet et al. 2002; Sigg 2006; Smith & Hughes 2008). Unfortunately, it was not possible to quantify the relative contribution of each of these components (e.g. using a general linear model) due to the restricted number of replicates (i.e. only five translocated populations). Nevertheless, this study provides pragmatic evidence that ~50 individuals should be considered the minimum number of founders to be used in macropods. In fact, as mentioned earlier, the only difference between the *Batalling* and StPI populations is the

number of individuals used to found the translocated population. Furthermore, the *Batalling* population suggests that a minimum population size of 3,000 individuals (Groom 2010) should be targeted in macropods, because it can ensure acceptable levels of genetic diversity within the time frame looked at by this research. This is confirmed by the fact that StPI, which has also an estimated population size of 3,000 (Groom 2010), had a significantly higher genetic variability when compared to the other two South Australian populations. Consequently, during the selection of new translocation sites it is essential to ensure that the site is capable to sustain a final population size above this minimum target and that threats limiting populations can be effectively managed. When this is not possible, ongoing management should be planned to optimise conservation outcomes by ensuring regular and effective supplementation.

When a translocated population is established, careful consideration should be given to the above-mentioned principles and, whenever possible, we strongly recommend the use of wild instead of captive-bred animals. Notwithstanding the potential to improve past practices, our data showed that genetic diversity has been seriously compromised in populations founded by a limited number of individuals and captive facilities rarely can house large number of individuals. Furthermore, the use of wild caught animals has been recommended to reduce the divergence of translocated populations (Smith & Hughes 2008) and it was demonstrated that the release of captive bred animals can have disadvantageous consequences even in supplementations because common alleles could dilute rare alleles in the target population (Sigg 2006).

Genetic investigations are equally important during the planning phase of a translocation (Stockwell et al. 1996; IUCN 1998) as well as to quantify and assess translocation success (Goossens et al. 2002). The genetic profiles of founders and of the re-introduced animals

should be evaluated in order to guarantee maximum diversity of potential breeders and to assess the impact and breeding success of introduced individuals (Goossens et al. 2002; Sigg 2006). A preliminary molecular investigation of the substructure and spatial organisation of the source population could provide valuable information in order to design a trapping regime that would optimise the representation of the source population. For example, in the *Perup* population, our molecular data have demonstrated that the probability that two woylies are unrelated was highest when they were trapped at a distance of at least 1-3 kilometres (Pacioni et al. Submitted).

In conclusion, the populations at *Karakamia* and *Batalling* are both good examples of the conservation value of translocations in that both still are valuable representations of the woylie genetic pool. Additionally, this study highlights the importance of a careful evaluation of critical factors that might affect each translocation phase: planning, execution and ongoing-management. Regular demographic and genetic monitoring can ensure early detection of potential problem and facilitate adaptive management measures in order to obtain the best possible conservation outcomes for the target species.

7.6 Acknowledgements

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8 An integration of genetic and demographic comparisons across populations and time reveals important insights of a species undergoing a rapid decline

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8.1 Abstract

Animal dispersal and population size fluctuations are interconnected factors that need to be understood if we are to correctly manage a species. Using data from 12 microsatellite loci, several genetic analysis techniques were employed in combination with trapping data (spanning over 30 years) to assess spatial structure and genetic bottlenecks of the last remaining indigenous woylie (or brush-tailed bettong, *Bettongia penicillata ogilbyi*)

populations. In doing so, we evaluated the performances of different molecular techniques in detecting the genetic changes associated with a rapid demographic decline. Considerations that arose from this study will aid those considering similar work. Spatial autocorrelation analyses proved to be highly informative in detecting differences of the spatial genetic structure between populations and confirmed that females are strongly philopatric, while the F_{ST}, Mean Assignment Index (mAIc) and Variance of Assignment Index (vAIc) tests had a reduced power. Among the bottleneck tests, the M-ratio approach was the most sensitive however the interpretation of its results would have been problematic in the absence of demographic data. Our results demonstrated that it is critical to consider time since demographic reduction and population dynamics to better interpret the results from such analyses. We also used the M-ratio for relative comparisons between populations and two temporal distinct sampling periods from the same population and showed that the Tutanning population did not recover from the post-European settlement decline, demonstrating that a relative comparison approach can be greatly informative.

8.2 Introduction

Identifying the causes of decline in wildlife is difficult, but necessary to determine appropriate management strategies to facilitate an effective and sustained recovery (Caughley 1994; Caughley & Gunn 1996). When identifying responsible risk factors, knowledge about the characteristics of the decline itself is critical (Caughley 1994; Caughley & Gunn 1996). Nevertheless, this knowledge is often subject to an in depth understanding of animal dispersal and demographics (e.g. population size fluctuations), which are inter-related population attributes.

Animal dispersal can increase reproductive success, avoid genetic inbreeding and be an effective strategy to cope with environmental changes (Banks *et al.* 2005). As well as being a key population attribute (Hawkes 2009), information about animal movements can be used to assess potential risks, such as disease transmission (Perkins *et al.* 2009), faced by a wild population.

From a conservation perspective, reduction in population size may have dramatic consequences. It can increase inbreeding (Frankham 1995b), which in turn will limit the evolutionary potential (Frankham 1996; Frankham *et al.* 1999), facilitate the expression of deleterious alleles (Ralls *et al.* 1988; O'Grady *et al.* 2006), and decrease fitness. For example, inbreeding may reduce the capacity of individuals or populations to efficiently respond to pathogens (e.g. Acevedo-Whitehouse *et al.* 2003; Charpentier *et al.* 2008). Moreover, density changes can directly or indirectly regulate dispersal events (e.g Sharp 1997).

Nevertheless, knowledge about dispersal and density changes is often not easily available. Timing as well as logistical and financial limitations could constrain further studies potentially needed to provide baseline information, especially when a species is declining. In these situations, molecular techniques are invoked as complementary, if not as an alternative, to former ecological methods (e.g. Sunnucks 2000). These techniques can provide inference about past and recent population dynamics, including dispersal, demographics and population size. They can also detect infrequent (but regular) events, that would otherwise go unnoticed with classic methods (Slatkin 1985).

We investigated dispersal patterns and fluctuations in population size in a small Australian marsupial, the woylie or brush-tailed bettong (*Bettongia penicillata ogilbyi*), that has recently undergone a dramatic decline. The woylie was upgraded to *Critically Endangered* in the IUCN

Redlist in 2009 in response to a decline of around 80% between 2001 and 2008 (Wayne *et al.* 2009). Interestingly, the woylie was the first Australian species to be removed from the Commonwealth and WA State Endangered species lists in 1996 (Start *et al.* 1998) as result of a strong recovery in response to the management initiatives that began in the 1970s such as baiting for feral foxes (*Vulpes vulpes*). Hence, the recent demographic relapse was unexpected and prompted a thorough assessment of the underlying population dynamics.

Current woylie distribution is limited to Western Australia and South Australia. Four genetically distinct indigenous populations exist, all in Western Australia (Pacioni *et al*. 2011). Two of these are in the Wheatbelt region: Dryandra Forest (Dryandra) and Tutanning Nature Reserve (Tutanning) and the other two within the Jarrah forest in Upper Warren: Kingston and Perup (Figure 8.1).

Limited ecological studies in the early 1980s suggested that woylie dispersal is possibly biased by gender (Sampson 1971; Christensen 1980). If this were correct, we would expect that females displayed a stronger geographical affinity whereas males would have presumably a less intense, if any, spatial genetic structure.

As such, this study aimed to (i) determine whether the indigenous populations have a spatial genetic structure and quantify its attributes; (ii) confirm sex-biased dispersal and quantify the distance covered during dispersal events; (iii) investigate whether there has been a change in the spatial genetic structure over time and (iv) improve our understanding of the demographic history of woylie populations. In doing so, we also evaluated the performances of different molecular analyses. In order to achieve these objectives we analysed genetic variation in 12 microsatellite loci, combined with demographic information obtained through trapping records spanning over 30 years. Our results are discussed in a conservation context

highlighting possible genetic consequences of the recent and rapid decline in this endangered marsupial.

8.3 Methods

8.3.1 Sample collection, microsatellites amplification and analysis

For this study, we used a subset of the genetic data published in Pacioni *et al.* (2011), which included all adults with complete information (gender and GPS coordinates. Table 8.1). This dataset was generated genotyping 12 microsatellite loci. Samples were collected in 2006 and were representative of the indigenous woylie populations at three different locations: Upper Warren (including two distinct populations: Kingston – from now on referred to as Kingston-06 – and Perup), Dryandra and Tutanning (Figure 8.1).

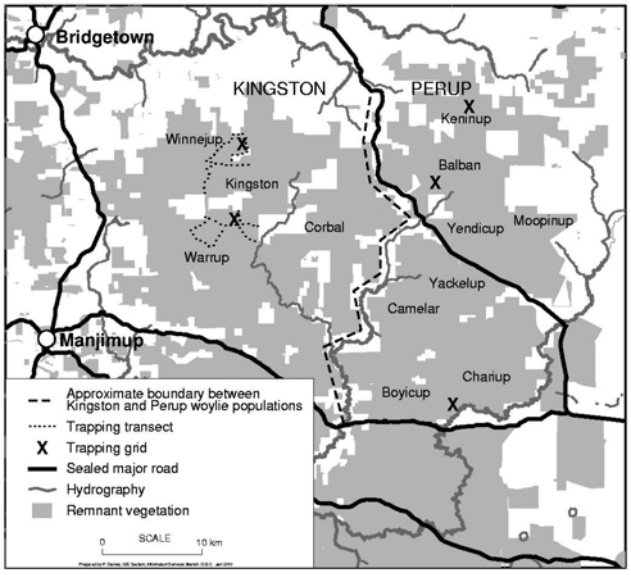


Figure 8.1 Forest blocks surveyed and described in the manuscript from the Upper Warren

In the Upper Warren region tissue samples were collected as part of two field components of the Woylie Conservation Research Project (see Wayne *et al.* 2006b). Trapping grids in five forest blocks were surveyed every two months: each grid consisted of 30 traps (5 lines x 6 traps) spaced 50 m apart. Trapping transects were also surveyed in these and additional six forest blocks twice per year: each transect was 10 km of forest tracks with 50 traps spaced 200 m apart.

Similarly designed transects were integrated with opportunistic trap points throughout the entire reserves in the other two locations (Dryandra and Tutanning). Both populations were at low density for the duration of the sampling, but while Dryandra was previously a high density population that declined between 2002 and 2005, Tutanning has, for the last 40 years, been approximately stable at low density (Sampson 1971; Groom 2010), except for a transitory density increase between 1984 and 1994 (Kinnear *et al*. 2002; Orell 2004).

An additional 22 tissue samples were collected in 1999 from the former Kingston transects ("Kingston-99", which crossed Warrup and Winnejup forest blocks. Figure 8.1) and preserved in 20% dimethylsulphoxide (DMSO) saturated with NaCl. DNA extractions and PCRs for these samples were carried out according to Pacioni and Spencer (2010). Data for the locus Y112 were not available for Kingston-99, so all comparisons that involved the use of this dataset were limited to 11 loci.

Table 8.1 Summary of the samples analysed in this study and measures of microsatellite variability.

Population	n	N_A (SE)	N_E (SE)	N_{AR} (SD)	H_E (SE)	Ho (SE)	P_{AR} (SD)
Dryandra	25	8.83 (± 0.89)	5.79 (± 0.63)	8.14 (± 2.66)	0.799 (± 0.03)	0.738 (± 0.05)	1.6 (± 1.94)
Tutanning	30	5.42 (± 0.62)	3.27 (± 0.33)	5.06 (± 1.7)	0.643 (± 0.05)	0.656 (± 0.08)	1.02 (± 1.2)
Perup	94	15 (± 1.81)	7.65 (± 0.9)	10.18 (± 2.98)	0.836 (± 0.03)	0.75 (± 0.04)	1.8 (± 1.97)
Kingston-2006	68	12 (± 1.33)	5.89 (± 0.61)	8.38 (± 2.51)	0.788 (± 0.04)	0.707 (± 0.06)	1.33 (± 1.28)
Warrup-Winnejup	42	9 (±0.85)	4.88 (±0.45)	7.57 (± 2.03)	0.759 (±0.04)	0.674 (±0.06)	1.48 (± 0.77)*
Kingston-1999	22	7 (± 0.87)	4.18 (± 0.55)	7.02 (± 2.51)	0.689 (± 0.06)	0.645 (± 0.07)	0.92 (± 0.74)*

n, number of individuals genotyped; N_A, average number of alleles; N_E, average effective number of alleles; N_{AR}, average allelic richness; H_E, expected heterozygosity; H_o, observed heterozygosity; *SE*, standard error; *SD*, standard deviation; P_{AR}, average private allelic richness. Warrup-Winnejup: woylie trapped in only one session in Warrup and Winnejup blocks. *Comparison between Kingston-1999 and Warrup-Winnejup.

When comparing Kingston-99 and Kingston-06 to reveal a possible change over time in the overall genetic diversity, we considered a subset of Kingston-06 data that only included woylies trapped in Warrup and Winnejup (Figure 8.1) in order to control for the bias that would otherwise arise from the different geographic representation of the two temporally distinct datasets. To compensate for further sampling bias, we calculated the average allelic richness (N_{AR}) and average private allelic richness (P_{AR}) using the rarefaction method implemented in HP-RARE (Kalinowski 2005) based on 17 diploid individuals and then compared N_{AR} of each locus with the non-parametric Wilcoxon signed-rank test using SPSS v.15. The rarefaction method compensates for differences in sample size producing unbiased estimates of allelic richness. In a population experiencing a bottleneck, the loss of (rare) alleles is greater than the loss of heterozygosity, making the comparison between allelic richness estimates a more biologically meaningful test (Kalinowski 2004) than the traditional heterozygosity measures. Nevertheless, we also reported the observed (H_o) and expected heterozygosity (H_E) (Hartl & Clark 1997) as well as the average of the observed (N_A) and expected numbers of alleles (N_E) (Brown & Weir 1983), calculated using GENALEX 6.2 (Peakall & Smouse 2006). The interpretation and importance of these parameters for conservation purposes have been discussed elsewhere (Pacioni *et al*. 2011) and it will not be addressed here.

Conversion of data formats between softwares was done using the export function in GENALEX 6.2 (Peakall & Smouse 2006) and CREATE (Coombs *et al.* 2008).

8.3.2 Spatial analysis

Using trapping data, longer-range dispersals were detected by calculating the maximum distance covered by animals. We considered all the animals that were trapped multiple times (Upper Warren, 1994-2008: 13,228 entries for 1932 individuals; Dryandra: 415 entries for 81 animals; Tutanning: 224 entries for 38 individuals) and computed the straight-line distances for all the available trap points for the same animals.

Molecular data were analysed with the spatial autocorrelation (SA_C) analysis implemented in GENALEX 6.2 (Peakall & Smouse 2006). After grouping the pairwise geographic distances (i.e. the distance between all possible pairs of individuals) in classes, this method determines if there is a correlation between the pairwise genetic distances (Smouse & Peakall 1999) and the geographic distances within each distance class. 1000 bootstraps were used to calculate the confidence interval around the autocorrelation coefficients (r_{SAc}) and 1000 random permutations to test statistical significance as described in Peakall *et al.* (2003). It is important to recognise that r_{SAc} does not provide an indication of genealogical relationships (e.g. full sibs, half sibs, etc.) but is strictly associated with relatedness (Double *et al*. 2005). In order to assess statistical significance of the differences between: (i) the observed correlogram of each gender within each population and the null hypothesis (of no spatial autocorrelation), (ii) correlograms of the two genders within the same populations, (iii) correlograms of each gender across different populations and (iv) correlograms of each gender in the two temporal cohorts for Kingston (1999 and 2006), we used the heterogeneity test developed by Smouse *et al.* (2008). Following Smouse *et al.* (2008), we considered a significant "positive" spatial autocorrelation when the analysis indicated a positive and significant correlation between the geographic distance and the genetic distance (i.e. closer pairs within a specific distance class are significantly more related than random pairs).

The distance classes were designed to be biologically meaningful and of a reasonable sample size to produce sufficient statistical power in the analyses. In the spatial genetic analysis, the coordinates of the central trap point of the grid (250 m x 300 m) were attributed to all woylies trapped within that grid. In this way, when carrying out the autocorrelation analysis, the distance class 0 m (null class) represented animals trapped within a grid or in the same trap in a transect. Based on our ecological data, we set the following distance classes: 1 m to 1 km (i.e. the average maximum distance covered by most animals within their home range, Christensen 1980); 1 km to 3 km (i.e. encompassing ~3-4 home ranges); 3 km to 6 km (expected normal maximum dispersal distance); 6 km to 9 km (peripheral traps of the same transect or very close forest blocks); 9 km to 12 km (approximate distance between close forest blocks); 12 km to 18 km (distant forest blocks); 18 km to 40 km and 40 km to 60 km (the latter two constituting the whole range across Perup, the larger area studied).

When comparing the two temporal cohorts for Kingston (1999 and 2006) and any of the modern cohorts in Upper Warren *versus* Dryandra and Tutanning, the null and first distance classes (i.e. 0 and 1 m- 1 km) were merged together (because no grids were used in Kingston-99, Dryandra and Tutanning). We also evaluated two datasets for Kingston-06 and Perup: one with pooled transect and grid sampling and one with transects only. In this way, we were able to confirm that the results for the lower range of distance classes were consistent and not influenced by a higher number of animals trapped within short distance in the grid (data not shown). Consequently, we present here only results from the combined datasets (transects and grids). Lastly, because of the differences in geographical representation of Kingston-99 and Kingston-06, we evaluated a subset of Kingston-06, including samples collected from Warrup and Winnejup forest blocks. These forest blocks are geographically comparable to the area sampled in 1999. The results (data not shown) were comparable with what obtained with the whole Kingston-06 dataset and are not discussed any further.

Auxiliary to this, the Mantel test implemented in GENALEX 6.2 (Peakall & Smouse 2006) was used to determine the correlation (r_{mt}) between a gender-specific pairwise relatedness measure (Queller & Goodnight 1989) and pairwise geographic distances separately within each population. Statistical significance of Mantel tests was assessed with 1000 random permutations while the statistical difference between r_{mt} of each gender within each population was determined converting r_{mt} into z-scores and comparing their difference to the normal distribution (Fisher 1921).

Lastly, three alternative statistics were used to further investigate the strength of any potential sex biased dispersal. We quantified genetic differentiation with F_{ST} values (Weir & Cockerham 1984) between each gender. Individuals of the philopatric gender would be expected to be more related among each other than animals of the dispersing gender (Goudet *et al.* 2002). Differences in the Mean Assignment Index (mAIc) and Variance of Assignment Index (vAIc) were also tested. These tests are based on the assumption that dispersers are less likely to be assigned correctly (e.g. mAIc should be lower for the gender that disperses, Goudet *et al*. 2002). These three tests were executed in FSTAT v2.9.3 (Goudet 1995) and statistical significance was based on 1000 randomisations.

8.3.3 Detection of genetic bottlenecks

Evidence of population bottlenecks was investigated by testing for an excess in heterozygosity (Cornuet & Luikart 1996) and mode-shift (Luikart & Cornuet 1998), using the program BOTTLENECK (Piry *et al.* 1999). Due to the relatively small number of loci analysed (n = 12), a Wilcoxon sign-rank test was applied, as recommended by Piry *et al.* (1999). A mixed model of microsatellite mutation was assumed, with single step mutations accounting for 95% of all

mutation events, and a variance among multiple steps of 12, as suggested by Piry *et al*. (1999). Additionally, we also used the M-ratio method (Garza & Williamson 2001), which evaluates the ratio of the total number of alleles to their size range. M-ratio drastically decreases in the event of a bottleneck and recovers slowly afterwards. This test is anticipated to produce positive results for at least 100 generations post-reduction (Garza & Williamson 2001).We calculated the M-ratios (M) with M_P_VAL (Garza & Williamson 2001) and the M-critical value (M_C, 95^{th} lowest percentile of the distribution of M from 10,000 simulations of an equilibrium population) using Critical_M (Garza & Williamson 2001) to establish statistical significance.

M-ratio was also used for a relative comparison between datasets. The demographic history of Kingston population was unclear due to a lack of comparable abundance data between 1999 (when the trapping success - the proportion of woylie captures over the total number of available traps - was around 60%) and 2005 (when the trapping success was around 40%). Therefore, it was difficult to discriminate whether Kingston already declined and then partially recovered or was declining. If the Kingston population underwent a second bottleneck between 1999 and 2006, a lower M-ratio would be expected from animals trapped from the same sites (Warrup and Winnejup forest blocks) in 2006 compared to the Kingston-99 cohort.

A similar relative comparison could be informative also about Tutanning past demographic history. Demographic data indicates that in the last 40 years, the census size of the Tutanning population has been approximately stable at around 300 woylies (Sampson 1971; Orell 2004; Groom 2010) but the historical census size is unknown. Under the assumption that, prior to European settlement, Dryandra and Tutanning were both of similar size and had similar levels of genetic variability, one would expect that Dryandra and Tutanning M-ratios had dropped similarly as a result of the bottleneck between the 1920s and 1960s. After the establishment of the baiting program, Dryandra recovered quickly while Tutanning recovered transiently

between 1984 and 1994 (Kinnear *et al.* 2002; Orell 2004) and then stabilised at a census size of 300-350 individuals (Groom 2010). Accordingly, Dryandra M-ratio is expected to have partially recovered while Tutanning M-ratio has stagnated or perhaps decreased further. In these relative M-ratio comparisons, we evaluated each locus separately and calculated the overall balance (i.e. the number of loci whose M-ratio decreased minus the ones whose M-ratio increased).

Recommended mutation parameters were used to simulate the M-ratio at equilibrium (M_{eq}) and M_c: 0.2 for the proportion of one-step mutations (p_s), 3.5 as the average size of non one-step mutations (Δ_g) and a mutation rate (μ) of $5x10^{-4}$/locus/generation (Garza & Williamson 2001). It was assumed that the population census sizes (N_c) prior to European settlement were similar to those estimated at the peak density observed after the commencement of the fox control program, immediately prior to the most recent declines (i.e. a conservative and best available estimate of pre-European populations) (Groom 2010). Following Frankham (1995a), we approximated the effective population size (N_e) to be 10% of N_c. Tutanning pre-European settlement N_e was assumed to be similar to Dryandra. We investigated if the results would change when doubling the population mutation parameter ($\Theta=4N_e\mu$ for diploid genes where N_e is the effective population size and μ is the mutation rate) and/or removing loci without the recommended attributes (Garza & Williamson 2001; Williamson-Natesan 2005).

8.4 Results

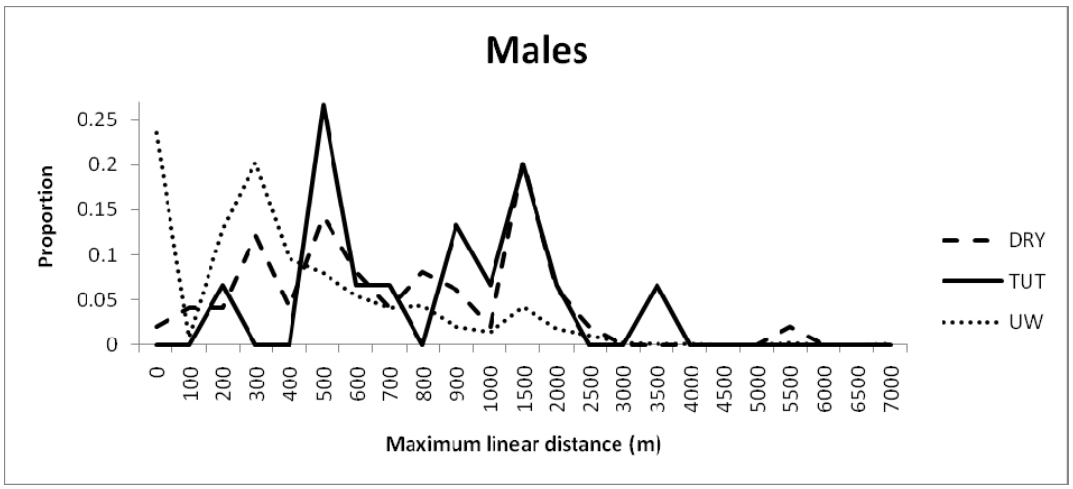
8.4.1 Genetic variability in the Kingston population

There was a marginally significant difference (p=0.05) in N_{AR} between samples collected from the Kingston sites in 1999 and in 2006 with N_{AR} being higher in 2006 (Table 8.1). It is notable that despite the small samples size, each locus contained, on average, 0.92-1.48 private alleles between the two time periods.

8.4.2 Spatial analysis

Based on the trapping records, males appeared to be more vagile than females. Across the four populations, in average, the maximum linear distances 76.2% (SD=14.1) of males were less than 1 km as opposed to 98.2% (SD=1.6) of females. Female distributions of maximum movements were broadly similar across populations (Figure 8.2). On the other hand, the distribution of male maximum movements appeared to be bimodal in Tutanning and Dryandra, while this was not the case in Upper Warren. The maximum linear distance moved was 6.9 km and 3 km for males and females, respectively (Figure 8.2).

a)



b)

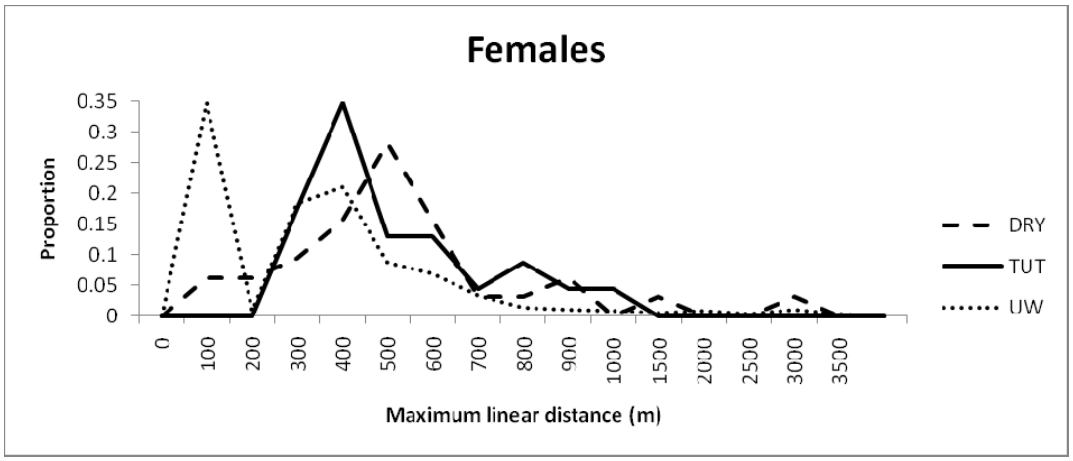


Figure 8.2 Proportion of maximum straight-line distances per locations within each gender

The analyses of molecular data confirmed through SA_C the positive spatial genetic structure for both genders in Upper Warren (Table 8.2), while Tutanning did not display any statistically significant spatial genetic structure ($p>0.1$; Table 8.2).

No difference was evident between the two genders within the same populations except in Perup ($p=0.036$, Table 8.3).

Kingston-06 had the strongest spatial genetic structure with both genders showing a significant positive r_{SAc} ($p<0.03$; Table 8.2) in the null and first three distance classes. Most of the other classes in Kingston-06 had a significant negative r_{SAc}. Additionally, in the comparison across populations of the spatial genetic structure in males, we observed a significant difference between Kingston-06 *versus* Perup ($p=0.001$; Table 8.4). Females revealed significant differences between Kingston-06 *versus* Tutanning and Perup ($p<0.02$; Table 8.4). Additional details of the SA_C results are summarized in Table 8.2, Table 8.3 and Table 8.4.

Of the woylies sampled in Kingston in 1999, only females showed an overall spatial genetic structure ($p=0.024$. Table 8.2). However, males had a similar trend to what was significantly evident in Perup. Additionally, males in the 1-3 km class demonstrated a statistically significant spatial genetic structure ($r_{SAc}=0.038$, $p=0.048$. Table 8.2). When each gender was compared separately with the respective Kingston-06 dataset, the tests for heterogeneity were not significant ($p>0.05$).

Table 8.2 Autocorrelation analysis and multiclass tests for each gender in each population conducted separately.

Interval (km)		0	0-1	1-3	3-6	6-9	9-12	12-18	18-40	40-60	ω	p value
Males												
Dryandra	r_{SAc}	NA	0.066	-0.003	-0.003	-0.110	NA	NA	NA	NA	23.68	0.006
	n	NA	17	26	39	9	NA	NA	NA	NA		
	p	NA	0.982	0.465	0.409	0.002	NA	NA	NA	NA		
Tutanning	r_{SAc}	NA	0.033	-0.022	0.027	NA	NA	NA	NA	NA	13.16	0.138
	n	NA	18	68	34	NA	NA	NA	NA	NA		
	p	NA	0.200	0.070	0.099	NA	NA	NA	NA	NA		
Perup	r_{SAc}	0.040	0.012	0.017	-0.008	-0.002	-0.009	-0.003	-0.008	-0.007	56.1	0.002
	n	115	89	186	214	452	138	161	427	48		
	p	0.001	0.135	0.006	0.076	0.280	0.106	0.266	0.008	0.169		
Kingston-2006	r_{SAc}	0.052	0.081	0.056	0.067	-0.020	-0.062	-0.032	NA	NA	88.73	0.001
	n	59	49	193	43	146	124	332	NA	NA		
	p	0.003	0.001	0.001	0.001	0.018	0.001	0.001	NA	NA		
Kingston-1999	r_{SAc}	NA	-0.009	0.038	-0.020	0.001	-0.057	NA	NA	NA	16.34	0.123
	n	NA	10	21	22	21	4	NA	NA	NA		
	p	NA	0.419	0.048	0.193	0.501	0.146	NA	NA	NA		
Females												
Dryandra	r_{SAc}	NA	0.131	-0.030	0.018	-0.071	NA	NA	NA	NA	21.01	0.028
	n	NA	7	19	20	9	NA	NA	NA	NA		
	p	NA	0.012	0.166	0.287	0.048	NA	NA	NA	NA		
Tutanning	r_{SAc}	NA	0.044	-0.016	0.007	NA	NA	NA	NA	NA	9.69	0.292
	n	NA	13	49	29	NA	NA	NA	NA	NA		
	p	NA	0.156	0.137	0.369	NA	NA	NA	NA	NA		
Perup	r_{SAc}	0.104	0.104	0.014	0.015	0.004	-0.016	-0.023	-0.037	NA	64.05	0.001
	n	42	15	29	66	123	36	82	135	NA		
	p	0.001	0.001	0.232	0.104	0.334	0.192	0.008	0.001	NA		
Kingston-2006	r_{SAc}	0.068	0.170	0.084	0.139	-0.001	-0.068	-0.071	NA	NA	76.1	0.001
	n	17	22	47	11	32	47	100	NA	NA		
	p	0.030	0.001	0.001	0.002	0.497	0.001	0.001	NA	NA		
Kingston-1999	r_{SAc}	NA	0.042	-0.002	0.059	-0.075	0.097	NA	NA	NA	27.22	0.024
	n	NA	9	13	3	9	2	NA	NA	NA		
	p	NA	0.068	0.471	0.151	0.002	0.127	NA	NA	NA		

r_{SAc}, corrected autocorrelation coefficient; n, number of pairwise comparisons; p, p values; NA, Not Available; ω, Multiclass test criterion.

Table 8.3 Single-class and multiclass test criteria with relative p values for comparisons between genders within the same populations

Interval (km)		0	0-1	1-3	3-6	6-9	9-12	12-18	18-40	40-60	ω	p value
Dryandra	t^2	NA	0.877	0.228	0.258	0.554	NA	NA	NA	NA	5.68	0.671
	p	NA	0.320	0.649	0.621	0.453	NA	NA	NA	NA		
Tutanning	t^2	NA	0.035	0.028	0.195	NA	NA	NA	NA	NA	1.49	0.956
	p	NA	0.852	0.868	0.643	NA	NA	NA	NA	NA		
Perup	t^2	6.124	6.329	0.005	1.341	0.127	0.077	1.013	2.649	0.838	29.78	0.036
	p	0.010	0.015	0.942	0.257	0.789	0.798	0.335	0.086	0.519		
Kingston-2006	t^2	0.141	4.722	0.835	1.856	0.342	0.044	2.519	NA	NA	18.77	0.167
	p	0.735	0.037	0.382	0.176	0.584	0.872	0.090	NA	NA		
Kingston-1999	t^2	NA	0.505	0.421	0.999	1.240	2.555	NA	NA	NA	11.96	0.286
	p	NA	0.493	0.567	0.306	0.259	0.114	NA	NA	NA		

t^2, Single-clasas criteria; ω, Multiclass test criterion; p, p values; NA, Not Available.

Table 8.4 Single-class and multiclass test criteria with relative p values for comparisons across the four populations for the same gender.

Interval (km)				0-1	1-3	3-6	ω	p value
Males								
Dryandra	vs	Tutanning	t^2	0.605	0.312	0.945	4.93	0.558
			p	0.428	0.592	0.336		
	vs	Perup	t^2	0.323	0.089	0.007	0.73	0.992
			p	0.770	0.902	0.998		
	vs	Kingston-2006	t^2	0.000	0.946	1.369	4.31	0.634
			p	0.996	0.438	0.266		
Tutanning	vs	Perup	t^2	0.008	0.515	0.417	1.37	0.969
			p	0.967	0.692	0.753		
	vs	Kingston-2006	t^2	0.380	2.615	0.641	8.11	0.226
			p	0.634	0.051	0.536		
Perup	vs	Kingston-2006	t^2	5.553	5.696	12.780	29.12	0.001
			p	0.017	0.014	0.002		
Females								
Dryandra	vs	Tutanning	t^2	1.137	0.070	0.059	3.42	0.754
			p	0.287	0.782	0.804		
	vs	Perup	t^2	0.080	0.273	0.002	1.20	0.972
			p	0.811	0.683	0.992		
	vs	Kingston-2006	t^2	0.005	2.559	2.603	9.05	0.173
			p	0.948	0.107	0.107		
Tutanning	vs	Perup	t^2	0.778	0.258	0.020	2.75	0.829
			p	0.400	0.669	0.944		
	vs	Kingston-2006	t^2	1.708	4.478	5.383	19.24	0.006
			p	0.190	0.025	0.014		
Perup	vs	Kingston-2006	t^2	0.345	2.753	7.958	15.33	0.017
			p	0.552	0.106	0.008		

t^2, Single-class criteria; ω, Multiclass test criterion; p, p values; NA, Not Available.

The Mantel tests showed a significant negative correlation between relatedness and the geographical distance in both Upper Warren populations with females always having a significantly higher r_{mt} than males (Table 8.5; Perup, p<0.005; Kingston, p=0.032). No significant correlations were found in Tutanning, while in Dryandra, differently from SA_C, a significant correlation was demonstrated only for males (Table 8.5).

Table 8.5 Correlations (Mantel tests) between Queller and Goodnight (1989) relatedness and geographical distance.

FEMALES	r_{mt}	p	MALES	r_{mt}	p
Dryandra	-0.075	0.263	Dryandra	-0.192	0.044
Tutanning	-0.014	0.462	Tutanning	0.108	0.116
Perup	-0.414	0.001	Perup	-0.094	0.017
Kingston	-0.396	0.001	Kingston	-0.283	0.001

Given the above results and the evident differences between locations, the test for difference in F_{ST}, mAIc and vAIc was carried out for three different datasets: the two Upper Warren populations, the three positive populations in the SA_C analysis (Perup, Kingston-06 and Dryandra) and all four "modern" populations concurrently. Interestingly, F_{ST} and mAIc tests were marginally significant when Perup, Kingston-06 and Dryandra were tested simultaneously while with the complete dataset, only the mAIc test was significant (Table 8.6).

Table 8.6 F_{ST}, Mean Assignment Index (mAIc) and Variance of Assignment Index (vAIc) tests of sex-biased dispersal

Test	Upper Warren	Upper Warren & Dryandra	All
F_{ST} Males	0.049	0.074	0.089
F_{ST} Females	0.066	0.094	0.113
p	0.062	0.048	0.1
mAI Males	-0.43	-0.47	-0.42
mAI Females	0.78	0.83	0.69
p	0.073	0.044	0.046
varAI Males	22.92	21.84	20.18
varAI Females	25.09	23.15	21.5
p	0.643	0.633	0.693

8.4.3 Detection of genetic bottlenecks

None of the tests for heterozygosity excess and mode-shift were significant. The M-ratio method, however, detected a reduction in population size in all populations except for Perup. When altering the parameters for the analysis (doubling the population mutation parameter and/or removing loci without the recommended attributes, see Methods), no difference was found for Tutanning and Kingston-99, while inconsistent results were obtained for Dryandra and Kingston-06 (data not shown).

There was an even balance between the number of loci that increased and the ones that decreased when the M-ratios were compared between Kingston-99 with Warrup and Winnejup in 2006 as distinct to the M-ratio comparison between Dryandra and Tutanning, which showed a decreased value in eight loci in the latter.

8.5 Discussion

8.5.1 Genetic variability in the Kingston population

The comparison of genetic variability parameters did not provide any additional information on the demographic history of Kingston. Given the available but incomplete evidence from demographic data that Kingston had undergone at the least a minor decline between 1999 and 2005 and that the spatial genetic structure changed between 1999 and 2006 (see below), we hypothesize that these parameters were not sensitive enough to detect recent demographic changes within the timeframe considered in this study. It is likely that, in a few generations, a genetic signal of the demographic changes in the population will be more evident.

8.5.2 Spatial analysis

There is overwhelming genetic evidence that both males and females have a positive spatial genetic structure. Females are strongly philopatric as shown by SA_C analysis, Mantel tests, F_{ST} and mAIc. It would appear that the dispersal range is less than 1 km for females and in average between 1 and 3 km for males. These genetically-derived findings are strongly supported by the comparable trapping data. Assuming an approximate radial home range of 0.6 km, based on radio-tracking estimates (23-35 ha, Sampson 1971; Christensen 1980), females are likely to be settling within or adjacent to their mother's home range. Males appear to typically disperse beyond multiple home range equivalents. This pattern is consistent with other bettongs including the rufous bettong, *Aepyprymnus rufscens* (Johnson & Payne 2002) and northern bettong, *B. tropica* (Pope *et al.* 2005). The analysis of trapping data showed that woylies are

capable of covering long distances and that these events are rare but not unique (Pacioni *et al*. 2011). Long distance movements (up to 6 km) were also detected in male boodies (burrowing bettong), *B. lesueur* and male rufous bettongs (Parsons *et al*. 2002; Pope *et al.* 2005). The inability of F_{ST}, mAIc and vAIc to consistently detect the sex bias is probably related to the statistical limitations of these tests, which are discussed elsewhere (Goudet *et al.* 2002).

All woylies that moved more than 3 km were adult males. These findings are therefore consistent with the hypothesis that subadult bettongs do not disperse large distances (Pope *et al.* 2000). However, a thorough assessment of the possible age of dispersal using trapping data is limited given that 95.8% of individuals were trapped for the first time as adults.

Remarkably, Tutanning is clearly different from the other populations having no spatial genetic structure. This suggests that woylies in Tutanning have to move more to find a suitable area where to settle. This hypothesis is partially supported by trapping data indicating that only ~7% of males in Tutanning moved less than 500 m (Figure 8.2). High population turnover, possibly associated with lack of suitable refugia from predators due to land clearing for agriculture (Sampson 1971), could be responsible for this pattern. Similarly, the dispersal of the yellow-footed rock-wallabies (*Petrogale xanthopus*) has been associated with social turnover and mortality (Sharp 1997). If this hypothesis is confirmed, then the lack of spatial genetic structure in Tutanning is due to long term population dynamics and Dryandra could incur to a similar destiny unless the population recovers. In this regard, it is interesting to note that both Tutanning and Dryandra showed a similar bimodal distribution of the linear maximum distances derived from the trapping data, possibly indicating that similar dynamics are present in the two populations.

Kingston-06 had the strongest spatial genetic structure (up to 6 km). We argue that the lack of similar structure in Kingston-99 is indicative of a biologically meaningful difference and that if more samples from 1999 were available, this could have become statistically evident (i.e. the statistical comparison lacked of power because of the limited sample size of the 1999 dataset). Males may have recently extended their genetic "signal" in Kingston given that males in the long distance class (3-6 km, r_{SAc} =0.67) were on average more related in 2006 than in 1999 (r_{SAc} =-0.02). We argue that this finding is a result of the population decline that occurred between 1999 and 2006, based on the incomplete trapping records. It is possible that groups of related animals colonised contiguous areas, which became free as a result of the decline, increasing the extent of their genetic "signature". It is important to note that, if this is correct and the difference in the genetic structure is a consequence of the change in the post-decline dynamics, the wider spatial genetic structure may be due to increased survival of animals that dispersed rather than increased dispersal distance. Christensen (1980) observed an increased survival of sub-adult males when a neighbouring patch of forest would become available, for example consequently to a bush fire. This phenomenon may be reinforced by additional ecological consequences of the low density. For example, reproductive dominance of a few animals, which may be sustained by their offspring in subsequent generations, would maintain the ancestor's genetic "imprinting" in the group (Storz 1999). We further envisage that, if the population density in Kingston increases and recovers to some extent, the current spatial genetic structure would become instable due to an increased frequency of incursions from neighbouring patches. This would result in a reduction of the strength of the spatial genetic structure, comparable to what observed in Perup.

8.5.3 Detection of genetic bottlenecks

No evidence of a bottleneck was detected in this study using heterozygosity excess and mode shift analytical approaches. The M-ratio method detected reductions in all population sizes except Perup. Interpretation of contrasting results obtained for Dryandra and Kingston-06 under different settings (doubling Θ and/or removing specific loci) was substantially improved by evaluating the results of the genetic analysis in conjunction with the demographic evidence derived from trapping data. Various factors may affect similarly the performances of these three analyses, including the time since the bottleneck (Cornuet & Luikart 1996), the nature of the post-decline recovery (i.e. demographic growth after post-reduction could create a heterozygosity deficiency which would balance out the heterozygosity excess; Cornuet & Luikart 1996; Busch *et al*. 2007) and migration (Cornuet & Luikart 1996; Luikart *et al.* 1998), which, at least in Upper Warren, could have replaced missing alleles (Pacioni *et al*. 2011). Additionally, the resolution power of the mode shift approach is limited when Θ is large (Williamson-Natesan 2005). These factors considered, it is highly likely that the timing of the sampling was too close or contemporary to the declines in Perup, Kingston and Dryandra to be detected by the heterozygosity excess (method that requires a minimum of 0.01-0.25 times $2N_e$ generations since the demographic reduction to have a power of more than 0.6-0.8; Cornuet & Luikart 1996) and M-ratio methods (Williamson-Natesan 2005). Consequently, we argue that the results of the M-ratio analyses are an outcome of the bottleneck in the 20th Century and the variability of the results observed between Kingston-06 and Dryandra and the neutral result of the comparison of M-ratios of the two sampling periods from Kingston (in 1999 and 2006) are an outcome of the demographic recovery that occurred between 1970 and 1998. The relative comparison of M-ratios was greatly informative for Tutanning, suggesting that this population did not recover from the bottleneck in the 20th century and since then has been affected by genetic drift.

8.6 Conclusion

The molecular methodologies applied in this study demonstrated mostly to be a valid approach when investigating population dynamics associated with a rapid decline. Sex-biased dispersal in woylies was demonstrated and its attributes characterised. SA_C analysis proved to be a very useful tool providing much higher resolution than the most commonly-employed methods. Subtle differences in genetic patterns within and between populations were detected, such as the spatial genetic structure of Kingston-06. This study further emphasized the benefits of the SA_C analysis and its recently enhanced statistical tests (Peakall *et al.* 2003; Double *et al.* 2005; Smouse *et al.* 2008), and many studies that involve different aspects of spatial ecology can greatly benefit from its use.

This study also provides clear empirical evidence of the practical limitations of several molecular approaches. Without demographic and ecological baseline data, it would have been extremely challenging to correctly interpret the differences in spatial genetic structure between populations. Furthermore, the results of bottleneck tests would have been equally difficult, thus emphasizing the importance of considering how pre-bottleneck conditions and timing of sampling can influence test performances. The integrated approach presented here between molecular and ecological techniques has greatly improved our understanding of woylie population dynamics. The overall results from this study reinforces the general contention (e.g. FitzSimmons *et al.* 1997; Double *et al.* 2005) that molecular techniques and established ecological methodologies can complement each other to provide an excellent platform for studying a species' ecology.

8.7 Acknowledgements

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9 Population viability analysis of woylies in Upper Warren, Western Australia

"Algebra has a seductive beauty of its own; it can lure him [the ecologist] away from the rough prose of population into elegant poetry of probability. […]The conclusion expressed in terms of the animals themselves is invariably dishevelled compared with algebraic conclusion, but the animals rather than the mathematics are the subject of study and the conclusion must be biological, not mathematical." (Caughley 1977, p.2)

9.1 Introduction

Population viability analysis (PVA) is classically defined as a technique to quantify the probability of extinction of populations (Boyce 1992; Morris & Doak 2002). However, the estimation of the risk of extinction is only one aspect of the potential use of PVA, which includes, among others, the comparison of threatening processes between different populations and the determination of key factors driving population dynamics (Lindenmayer et al. 1993a; Morris & Doak 2002). It also contributes towards a quantitative risk assessment (Haydon et al. 2002; Armstrong et al. 2003), the establishment of research priorities and aims of monitoring programs and the evaluation of different management strategies (Lindenmayer et al. 1993a; Starfield 1997; Morris & Doak 2002).

The implementation of PVA in wildlife research and management has not always been received favourably (Lindenmayer et al. 1993a; Bart 1995). For example, the accuracy of a model is jeopardised by the uncertainty of the parameters that determine the outputs and the assumptions implied in the model itself (Lindenmayer et al. 1993a; Bart 1995; Lacy 2000). The suitability of the typology and assumptions of models need to be assessed in view of the purpose of their use (Lindenmayer et al. 1993a; Bart 1995; Starfield 1997; Lacy 2000). To be reliable, they have to be critically reviewed and the conclusions extrapolated from the model must have a sound biological meaning (Lindenmayer et al. 1993a; Bart 1995; Caughley & Gunn 1996; Lacy 2000). Bart (1995) formalised the review process, establishing four criteria for acceptance of models in wildlife conservation. These include: i) *Structure of the model* - a full disclosure of the model structure and underlined assumptions to evaluate their compatibility to the biology and ecology of the species under study (see also Lindenmayer et al. 1995; Starfield 1997). ii) *Parameter values* - a thorough analysis of the consequences of possible measurement errors (also defined as sensitivity testing, Lacy 1993; Starfield 1997; Lacy 2000). iii) *Secondary predictions of the model* - Evaluation of secondary, non standard outputs of the model. iv) *Primary predictions of the model* - validation of the model comparing the predictions with real data (but notably Starfield (1997) considered this implicit in the validation of the assumptions).

Clearly, the weaknesses of PVA are considerable when there is a substantial lack of data. However, often alternatives do not offer a higher confidence in the appropriateness of the conclusions and, if a decision needs to be taken, at least PVA represents a tool capable of quantifying the extent of the lack of knowledge (Lindenmayer et al. 1993a; Starfield 1997). Moreover, new technology improvements and gained experience facilitate the correct use of this relatively modern approach as demonstrated by a recent review (Brook et al. 2000).

In this study, PVA was used to investigate the relative effects of different variables (small population size, feral predation, baiting program and inbreeding) to the population demography and genetic diversity of woylies. Additionally, an estimate of the minimum population size and an approximate threshold mortality rate that would lead to a high likelihood of extinction were inferred. Lastly, it was hoped that the results would indicate the direction of further research, and that future development of the model would lead to quantification of risks and enable testing of different management strategies.

9.2 Materials and Methods

VORTEX 9.96 (Lacy et al. 2003) was used to model genetic and demographic dynamics associated with different scenarios. VORTEX is an individual-based model which has the advantage of accommodating different processes and, most significantly, examining their interactions, therefore, overcoming one of the most important limitations of state-variable models (Huston et al. 1988; Lacy 2000). Moreover, individual-based models do not require simplified assumptions to describe complex phenomena that could incur the risk of over or under estimating the probability of extinction, as well as other population parameters (Huston et al. 1988; Lacy 2000). VORTEX was also chosen on the basis of its potential, in future developments, to incorporate the demographic analysis with epidemiological or spatially-explicit models, since this software can be integrated with other softwares, for example OUTBREAK and SPATIAL (Miller & Lacy 2005). Such additions would maximise the power of the viability analysis technique (Huston et al. 1988; Letcher et al. 1998; Hawkes 2009).

9.2.1 Assumptions

Despite the fact that fewer assumptions are required for individual-based models, some are still needed and, in VORTEX, these are mainly related to facilitate the program flow and have been described in detail elsewhere (Lacy 1993). None of them was considered to cause major limitations to the development of "biologically sound" outcomes from the simulation analysis. However, the assumptions of i) indiscriminate (i.e. not selective) additional mortality imposed when populations reach the carrying capacity and ii) equal mortality and reproduction probability of any of the adult ages are unlikely to be met. It would be expected that weaker, less dominant or experienced animals would have restricted access to resources when these are not easily available. Similarly, it would be expected that mortality and reproduction is distributed differently in various ages of adulthood. The discrepancies generated from these assumptions and the "real" dynamics would most likely overestimate the effective population size and, consequently, the simulations would slightly overestimate the final genetic diversity. Similarly, the fact that VORTEX by default models only one locus with a number of alleles equal to two times the initial population size most likely causes moderate increased genetic diversity estimations. However, relative differences between different scenarios should be of the same magnitude. Also, due to a lack of detailed information, no seasonal differences were taken into account although different weather conditions are likely to influence survival and food availability.

9.2.2 Model parameters

The VORTEX time-unit (the length of each cycle) was set to 91 days (hereafter time-unit). This appropriately accommodated woylie age stages. At apprximately 100 days, joeys leave the pouch and stay at foot up until 180 days. Females start reproducing between 180 and 240 days

of age, while males start after 270 (Sampson 1971; Christensen 1980). Consequently, for modelling purposes, females can be considered adults after two time-units (i.e. age 1 (first time-unit after birth) = pouch young; age 2 (second time-unit after birth) = juvenile; age 3 and afterwards = adult), while males, have an additional sub-adult stage, becoming adults after the third time-unit following birth (i.e. age 1 (first time-unit after birth) = pouch young; age 2 (second time-unit after birth) = juvenile; age 3 (third time-unit after birth) = subadult; age 4 and afterwards = adult). Baiting to control feral predators (mainly foxes) is carried out four times per calendar year (hereafter year) (Armstrong 2004). Hence, a 91 day time-unit also easily allowed the inclusion of a baiting program (i.e. one baiting session per time-unit).

Most population parameters were drawn from this study, published and unpublished literature (e.g. DEC reports) as well as field data collected during the WCRP (Table 9.1 and a list of abbreviations used is provided in Table 9.2).

Exponential growth rates (*r*) were calculated as the natural logarithm of the annual growth rate (λ, Caughley 1971):

$$r=ln(\lambda)$$

where $\lambda=N_{i+1}/N_i$ (with *i*, the *i*-th point in time at which the population size *N* is calculated and *i+1* the subsequent estimate in time).

When the exponential maximum growth rate (Caughley 1971; Caughley 1977) was calculated from trapping data (see below) the different times between trapping intervals needed to be taken into account (Morris & Doak 2002). This was achieved using the linear regression method described by Dennis et al (1991) implemented in a customised version of the

"Schlinger Tool G" (SCMG 2009) for Microsoft® Excel 2007, where the square root of the length of the time intervals ($\sqrt{(t_{i+1}-t_i)}$) was used as the independent variable. The density independency of the section of data under analysis was confirmed through a linear regression analysis with $\alpha=0.05$ in the "Schlinger Tool G" (SCMG 2009). No significant correlation between the changes of *r* and the population size over time was expected, because in the definition of the maximum growth rate it is implied that there is no limitation in the resources available to individuals of the population (Caughley 1971; Caughley 1977).

Table 9.1 Details of settings in VORTEX 9.96 for the baseline scenario of PVA in a woylie population in Upper Warren.

Category	Parameter	Value
Scenario Settings	Number of Years	400 time-units (i.e. 100 years)
	Time-unit	91 days
	Extinction Definition	Only one gender remains
	Number of Populations:	1
	EV Concordance of Reproduction and Survival	Yes
	Number of Types of Catastrophes	0
Reproductive System	Monogamous/Polygynous/...:	Polygynous
	Age of First Offspring for Females	2 time-units
	Age of First Offspring for Males	3 time-units
	Maximum Age of Reproduction	36 time-units
	Maximum Number Brood/time-unit	1
	Maximum Number of Progeny per Brood	1
	Sex Ratio at Birth	1:1
	Density Dependent Reproduction	$(89.3-[(89.3-57)(N/K)^{16})]N/(0.1+N)$
Reproductive Rates	% Adult Females Breeding	89.3
	EV % Breeding	3.5*
	Specify Exact Distribution	25-0/75-1
Mortality Rates	Mortality of Females as %: 0-1 time-units	4.4
	EV Mortality of Females as %	0.4
	Mortality of Females as %: 1-2 time-units	15
	EV Mortality of Females as %	1.5
	Mortality of Females as %: >2 time-units	3.9
	EV Mortality of Females as %	0.39
	Mortality of Males as %: 0-1 time-units	4.4
	EV Mortality of Males as %	0.4
	Mortality of Males as %: 1-2 time-units	10
	EV Mortality of Males as %	1
	Mortality of Males as %: 2-3 time-units	7
	EV Mortality of Males as %	0.7
	Mortality of Males as % >3 time-units	3.9
	EV Mortality of Males as %	0.39
Mate Monopolization	% Males in Breeding Pool	80
Initial Population Size	Start with:	Stable age distribution
	Initial Population Size: (All age classes)	300
Carrying Capacity	Carrying Capacity (K):	5000
	SD in K due to EV:	500

Additional scenarios were modified as indicated in Section 9.2. EV = environment variation; SD = standard deviation; * Three times this value would match the range of values found in the field.

Table 9.2 List of abbreviations used to identify model and population parameters in the PVA.

Symbol	Definition
%RecAl	Percentage due to lethal recessive alleles
EV	Environment variation
Ht	Final average expected heterozygosity across 100 iterations
i	*i*-th point in time of a time series
K	Carrying capacity: maximum number of individuals that a specific environment can sustain
LEs	Lethal equivalent
N	Population size: total number of individuals of a population
$N_{(ext)}$	Final average population size across 100 iterations after removal of populations that went extinct
$P_{(Ext)}$	Probability of extinction: proportion of populations that have become extinct in 100 iterations
P_0	Proportion of breeding females when the population is close to zero (See Materials and Methods)
P_F	Proportion of fecundity that apply for time-unit (See Methods)
P_K	Proportion of breeding females at carrying capacity (See Methods)
P_N	Proportion of breeding females when the population is N (See Methods)
P_S	Proportion of survival that apply for time-unit (See Methods)
r	Exponential growth rate: r=ln(λ)
$r_{(pop)}$	Population growth rate over the last 50 years of the simulation
$r_{(Rec)}$	Recovery rate: average *r* from the beginning of the simulation until *N* either stabilized or declined
SR	Uniform seeded random number generator function
λ	Annual growth rate: $\lambda=N_{i+1}/N_i$

9.2.2.1 Baseline scenario

A baseline scenario was created to describe demographic dynamics in the absence of inbreeding depression, as well as in the absence of the contribution of any additional mortality caused by feral predation and/or other stochastic events (e.g. bush fires). The maximum age of reproduction in the model was defined as the maximum life expectancy because it is thought that woylies reproduce throughout their life (Christensen 1980; Delroy et al. 1986). Life span in the wild was determined using trapping records from the Upper Warren (>26,000 woylie records spanning 34 years), where animals trapped for the first time were sexually immature (i.e. known age). Because woylie females can give birth to up to three young per year (Sampson 1971; Christensen 1980), to adjust for the 91 day time-unit, 75% breeding females were set as having one pouch young and 25% none.

Karakamia manifested a seasonal reduction in the proportion of breeding females, suggesting that once the carrying capacity is reached, there is self-regulation of the reproductive output (Ward et al. 2008a). This is also supported by observations on Venus Bay Island A (South Australia): when the population size reached its maximum values the proportion of breeding females dropped to 47% (Delroy et al. 1986). Consequently, the reproductive success was modelled as density dependent using the formula:

$P_N=(P_0-[(P_0-P_K)(N/K)^B])N/(N+A)$ (Miller & Lacy 2005)

Where N is the population size, P_N is the proportion of breeding females when the population is N, P_K is the proportion of breeding females at carrying capacity and P_0 is the proportion of breeding females when the population is close to zero. The parameter B describes the effect that the density has on the reproductive success and A is the Allee effect.

An average of 89.3% of females were found with pouch young at any point in time in Upper Warren (Christensen 1980) and this finding was confirmed during field work in 2006 (Ward et al. 2008a). This value was used as P_0, while 57% was used for P_K based on Karakamia estimations.

The value B=16 and A=0.1 were arbitrarily chosen so that the density effect was limited at the extreme of the distribution – very low and very high density (Miller & Lacy 2005).

Trapping data were used to determine woylie exponential growth rates and indirectly juvenile and subadult mortality rates. Trapping success (the proportion of woylie captures over the total number of available traps) are considered to adequately represent woylie population size (Wayne et al. 2006b). However, a few issues may arise from their use. “Trap learning” is likely

to initially increase the proportion of trappable individuals within the population and generate a significant bias. Discarding the data from the first year of trapping (4-6 sessions) was considered sufficient to adjust for this bias. Moreover, density of catches per trap has been suggested as a better demographic index rather than trapping success, because it adjusts for the reduction of trap availability over time during the trapping sessions (Caughley 1977). Unfortunately, the database was incomplete and did not allow the calculation of this adjusted parameter. However, it was demonstrated that typically, among the sympatric species, woylies are responsible for trap saturation. This means that woylies are the species that usually restricts trappability of other species, rather than being the one limited by other sympatric species (Wayne et al. 2008a). Furthermore, for the purpose of this analysis, the first years were the most relevant (see below) and, in these years, trap availability was considerable (because absolute trapping success in woylies as well as other species were low) and the few traps unavailable are unlikely to cause a major bias in the growth rate estimations.

Since fox control by baiting in Upper Warren, began in the 1970s (see Chapter 1) woylie populations have shown a substantial growth rate (Orell 2004). The maximum values of exponential growth rate calculated was used as an approximation of the intrinsic growth rate in the model (as defined by Caughley 1971; Caughley 1977), which was then used to approximate the mortality rate of juvenile and subadult age classes as explained below.

The only available estimation of mortality rates for different age classes was carried out in the presence of foxes in Upper Warren and with very limited fox control (Christensen 1980). A direct quantification of the additional mortality caused by foxes was not available. However, Christensen (1980) reported that more than 50% of mortality cases were caused by foxes. A similar proportion were reported also in other studies of small marsupials (Freegard 2000; Short et al. 2002). Consequently, Christensen's (1980) mortality rates were halved and used as

starting values for adults and pouch young age classes in the baseline scenario (which models the population dynamics in the absence of predation; Table 9.1). Mortality rates for juveniles and subadults were progressively reduced until the maximum per year exponential growth rate generated by the model matched the observed exponential maximum growth rate (Table 9.3). Juvenile and subadults are the age classes whose survival rates are more difficult to establish as a consequence of their reduced trappability, dispersal - which occurs mainly at this age (Christensen 1980; Pacioni et al. Submitted), and the rapidity at which animals reach the following age classes (Sampson 1971; Christensen 1980; Ward et al. 2008a). Additionally, in woylies as well as in other macropods, these classes are considered to be more affected by predation (Christensen 1980; Spencer 1991; Freegard 2000) and consequently Christensen's (1980) mortality rates for juveniles and subadults are, among the different age classes, most likely being overestimated when used in the baseline scenario (in which no predation is modelled).

Table 9.3 Summary of mean exponential maximum growth rate calculated from transects in Winnejup and Warrup forest blocks.

Year	Winnejup	Warrup
1994	1.49 (0.4)	2.29 (0.44)
1995	0.92 (0.05)	0.42 (0.74)
1996	0.32 (0.94)	0.14 (1.69)
1997	0.35 (0.09)	0.34 (0.02)
Mean 95-97	0.53 (0.34)	0.3 (0.15)

	Combined transects	***PredYesBait100***
Mean 95-97	0.41 (0.27)	0.49 (0.26)

Standard deviation is reported between parentheses. Recovery rate($r_{(Rec)}$) calculated from the model with predation and baiting included (*PredYesBait100*) is also reported.

Following broadly accepted recommendations (Lacy 1993; Bart 1995; Starfield 1997; Lacy 2000), several population parameters were altered to investigate the influence that these have on demographic projections (this is referred to as sensitivity testing). Mortality rates for all age classes were tested independently, progressively increasing the baseline values up to six fold. Additionally, different carrying capacity values and initial population sizes were tested. Among the reproductive parameters, only the percentage of breeding males (60%, 90% and 100%) was tested because of the confidence in the other reproductive variables due to extensive and long term field data collection (Sampson 1971; Christensen 1980; Delroy et al. 1986) and investigations carried out on both captive and wild woylie populations (Smith 1992, 1994, 1996; Johnson & Delean 2001).

9.2.2.2 Additional modelling scenarios

Several scenarios were also modelled to investigate the effects that different processes would have on projections of population demography and their interactions (Table 9.4).

Predation by introduced foxes and cats is a significant component of mortality in all age classes (Freegard 2000; Short et al. 2002), so scenarios that included predation by introduced predators with and without the mitigation effect of the fox-baiting program were incorporated into the model to investigate how these influence population dynamics. In Upper Warren, as well as in most other locations where woylies occur (e.g. Batalling State Forest, Tutanning Nature Reserve, Dryandra woodland), fox baiting is carried out under the Western Shield program (Armstrong 2004; Wyre 2004). Efficiency of the baiting is influenced by the timing of the bait distribution, weather conditions (rain dilutes sodium monofluoroacetate – the active ingredient of the fox baits – reducing the doses ingested) and prey availability (Saunders et al. 2000). No quantification of the efficacy fluctuations was available because this is monitored

through indirect evidence based on mammal abundance (Armstrong 2004; Orell 2004; Wyre 2004). The modelling of the variable efficiency of the baiting program was achieved using the catastrophe option available in VORTEX, which enables one to proportionally reduce the survival rates and fecundity. Initially, Christensen's (1980) data were used to quantify the effect of predation in absence of the baiting program. Given the rationale that foxes do not necessarily kill woylies each time they attack, but they are likely to cause pouch young ejection, not only an additional mortality but also a reduction in fertility was added, as shown below. Secondly, a random effect (using a uniform – i.e. not normally distributed – random function) was used to express the variability in baiting efficiency. When the random number was zero, the efficiency was 100% and there was neither additional mortality nor reduced fecundity. When the number was one, the baiting efficiency had no effect (0%) and the fecundity and mortality values changed in the same way as if the baiting was not carried out. Lastly, these parameters were included in a density dependent function.

Despite the presence of foxes, woylies did not disappear from Upper Warren, Dryandra and Tutanning for various hypothesised reasons. For instance, much of the sympatric native marsupial fauna consume plants of the *Gastrolobium* genus that contain sodium monofluoroacetate. Therefore, while native fauna have a higher tolerance towards the toxin, introduced eutherian predators were likely to have undergone secondary poisoning, providing protection to woylie populations (Christensen 1980). An alternative hypothesis, at least in the open woodlands, was suggested by Sampson (1971) who believed that the thick scrub offers protection from predation to woylies; however, as the population density increases, animals are forced into less optimal, open areas where they are quickly predated, limiting the population growth. The two hypotheses are clearly not mutually exclusive and possibly both phenomena were responsible for the survival of woylies (perhaps with a different proportional contribution depending on the area). Either way, woylie density would appear to be the driving

factor, consequently, the variable efficiency of the baiting program and decreased predation success at low population densities was modelled using the following final formulae:

$P_F=(1\text{-}SR\text{x}0.111)^{(N/K)}$

$P_S=(1\text{-}SR\text{x}0.45)^{(N/K)}$

P_F and P_S are the proportion of fecundity and survival for time-unit, respectively (i.e. the baseline values are multiplied for P_F and P_S). *SR* is a seeded random number generator function (between zero and one with uniform – i.e. not normally – distribution) in VORTEX, linked with the time-unit and iteration (Miller & Lacy 2005). In this way, both P_F and P_S obtain the same random number but it differs for each time-unit and iteration. *N* is the population size and *K* the carrying capacity.

Inbreeding depression was also modelled in separate scenarios. Inbreeding depression may become a significant component in reducing population fitness especially when the effective population size is small; therefore the investigation of its effect on the projections of population viability is recommended (Lindenmayer et al. 1995; Caughley & Gunn 1996; Lacy 2000; Allendorf & Luikart 2007). Because there are no studies on the number of lethal equivalents in wild woylie populations, the effect of a range of lethal equivalents from 3.14 to 12 with 1.57-4.8 lethal recessive alleles were explored as suggested by the literature (Ralls et al. 1988; Lacy 1993; Crnokrak & Roff 1999; O'Grady et al. 2006; Traill et al. 2009). VORTEX calculates two genetic diversity parameters: heterozygosity and number of alleles, and directly calculates inbreeding coefficients (Lacy 1993). Most of the time, it is not possible to directly calculate inbreeding coefficients from free range animals and often heterozygosity is used as an indirect index of inbreeding (e.g. Britten 1996; Frankham 1998; Charpentier et al. 2008;

Hansson & Westerberg 2008). Although, in a natural context this approach might present some drawbacks (Amos et al. 2001; Balloux et al. 2004; Hansson & Westerberg 2008), because VORTEX explicitly models genetic variability on a neutral locus, such limitations are not of concern for the simulation outcomes and heterozygosity is linearly related to inbreeding. Therefore, only heterozygosity was analysed in the results because it is considered a more "realistic" parameter and facilitates comparison between PVA and the genetic investigation conducted in woylies as well as other species. The effect of inbreeding depression will not be evident if the simulations are not run long enough to allow sufficient time to accumulate disadvantageous alleles (i.e. inbreeding depression is not important with high levels of genetic diversity while it will be evident with low genetic variability caused by genetic drift over time) (Allendorf & Luikart 2007). When inbreeding depression was included in the scenarios, the time "zero" was realigned with the time-unit when the population heterozygosity first fell below 80% and 64% heterozygosity, which were the approximated values found in the natural occurring populations (80% Dryandra and Upper Warren, 64% Tutanning; Pacioni et al. 2011).

The association between the processes of predation, baiting program and inbreeding were incorporated into the models and were analysed as a function of different carrying capacities (and indirectly, different average population sizes). All the scenarios started with a population size of 300 individuals except for the scenarios specifically dedicated to investigate outcomes from different initial population sizes.

Lastly, using the mortality rates of the baseline scenario as starting values, the mortality rates were gradually increased from an absolute, fixed amount in all age classes in order to establish the maximum level of mortality that could be sustained by a woylie population above which the probability of extinction was more than 50%.

Table 9.4 List of scenarios and relative parameters altered with respect to the baseline scenario

Scenario	Modified parameters from baseline scenario
baseline	See Table 9.1
baseline_K(500)	Carrying capacity (K)=500
baseline_K(1000)	Carrying capacity (K)=1000
baseline_K(2000)	Carrying capacity (K)=2000
Ad_Mor(100)	Adult mortality increased two fold
Ad_Mor(200)	Adult mortality increased three fold
Ad_Mor(300)	Adult mortality increased four fold
Ad_Mor(400)	Adult mortality increased five fold
Ad_Mor(500)	Adult mortality increased six fold
Juv_Sub_Mor(100)	Juvenile and subadult mortality increased by two fold
Juv_Sub_Mor(200)	Juvenile and subadult mortality increased by three fold
Juv_Sub_Mor(300)	Juvenile and subadult mortality increased by four fold
Juv_Sub_Mor(400)	Juvenile and subadult mortality increased by five fold
Juv_Sub_Mor(500)	Juvenile and subadult mortality increased by six fold
PY_Mor(100)	Pouch young mortality increased by two fold
PY_Mor(200)	Pouch young mortality increased by three fold
PY_Mor(300)	Pouch young mortality increased by four fold
PY_Mor(400)	Pouch young mortality increased by five fold
PY_Mor(500)	Pouch young mortality increased by six fold
%MalesBreed(60)	% Males in Breeding Pool (see 'mate monopolisation')=60%
%MalesBreed(90)	% Males in Breeding Pool (see 'mate monopolisation')=90%
%MalesBreed(100)	% Males in Breeding Pool (see 'mate monopolisation')=100%
InitPopSize(100)	initial population size=100
InitPopSize(500)	initial population size=500
PredNoBait(1)	Density dependent predation*, no baiting
PredNoBait(2)	Predation (not density dependent), no baiting
PredYesBait(100)	Density dependent predation, baiting program
PredYesBait(500)	Density dependent predation, baiting program, K=500
inb3K(500)	LEs=3.14, %RecAl=50%, K=500
inb3K(1000)	LEs=3.14, %RecAl=50%, K=1000
inb12K(500)	LEs=12, %RecAl=40%, K=500
inb12K(1000)	LEs=12, %RecAl=40%, K=1000
inb3_PredK(500)	LEs=3.14, %RecAl=50%, K=500, density dependent predation, baiting program
inb3_PredK(1000)	LEs=3.14, %RecAl=50%, K=1000, density dependent predation, baiting program
inb12_PredK(500)	LEs=12, %RecAl=40%, K=500, density dependent predation, baiting program
inb12_PredK(1000)	LEs=12, %RecAl=40%, K=1000, density dependent predation, baiting program
inb12_PredK(2000)	LEs=12, %RecAl=40%, K=2000, density dependent predation, baiting program

LEs = Lethal equivalent; %RecAl = Percentage due to lethal recessive alleles; * see Section 9.2 for details on the density dependent function.

9.2.3 Statistical analysis

To evaluate the effect of different model settings, 100 iterations were performed and the final average population size ($N_{(ext)}$, average population size after removal of populations that went extinct), final average heterozygosity (Ht) and probability of extinction ($P_{(Ext)}$, proportion of populations that have become extinct) were evaluated after 100 years. A length of 100 years was arbitrarily chosen in line with the IUCN time frame for classification of extinction risk (IUCN 2001). Because the number of data points was more than 30 (i.e. 100 data points, one for each iteration), a parametric test was considered robust (Pallant 2007; Field 2009) and the difference between population parameters generated from scenarios whose carrying capacity setting was equal, was assessed using the parametric t-test. The assumption of homogeneity of variance was confirmed with the F-test in Microsoft® Excel 2007. Similarly, to investigate the trend of the population growth, the average population growth rate ($r_{(pop)}$) over the last 50 years of the simulation (i.e. removing the initial growth at low density) was calculated and compared. Due to prohibitive computational time, statistical analyses were limited to a carrying capacity of 500 individuals when the starting level of genetic diversity (Ht) was 64%.

The recovery rate ($r_{(Rec)}$), defined as the average r from the beginning of the simulation until N either stabilized or declined – i.e. until the first difference $N_{i+1} - N_i$ was either null or negative – was calculated as an indication of the capacity of the population to recover from low density. The expected absence of a normal distribution of $r_{(Rec)}$ was confirmed with the Kolmogorov-Smirnov test. The recovery rate ($r_{(Rec)}$) for different scenarios were also compared to assess their relative differences using the non-parametric Wilcoxon signed-rank test using SPSS statistics ver 17 (SPSS inc.).

9.3 Results

9.3.1 Sensitivity testing

Sensitivity testing was conducted to analyse the influence of variations in the demographic parameters on the results of the PVA. The summary of the parameters altered and their influence on the results of the simulations are reported in Table 9.5.

The model was quite robust to changes in mortality rates in adult and pouch young age classes. No significant differences were found in $r_{(pop)}$ and $N_{(ext)}$, except for $N_{(ext)}$ when the adult mortality was increased by six fold relative to the baseline model (Table 9.5). Variation of mortality rates of juveniles and subadults had a substantial effect on $N_{(ext)}$, which was already significantly reduced by the five fold increases in the mortality rates of juveniles and subadults relative to the baseline model (Table 9.5; Figure 9.1). A substantial reduction of $r_{(Rec)}$ was observed with all modifications of the mortality rates within any of the age classes (Table 9.5). It is interesting to note that a change of pouch young mortality had the least effect while alterations of juvenile and subadult mortality rates caused, proportionally, the greatest effect. For example, when the juvenile mortality was increased by four fold a similar reduction of $r_{(Rec)}$ was not reached in any simulations where pouch young mortality rates were altered ($r_{(Rec)}$ was 53% less than in the baseline scenario) and it was more than the reduction obtained when the adult mortality was increased by five fold (Table 9.5). Genetic diversity, although it was statistically reduced, was not reduced to a biologically important proportion (less than 1%), except when the juvenile mortality was increased by six fold (*Juv_Sub_Mor(500)*), in which case there was a reduction of 53.7% compared with the baseline scenario (Table 9.5).

The other parameters tested (initial population size, percentage of males breeding and carrying capacity) did not appear to significantly influence $N_{(ext)}$, $r_{(Rec)}$, $r_{(pop)}$ or $P_{(Ext)}$ (Table 9.5).

9.3.2 Analysis

Predation had a significant effect on $N_{(ext)}$ (Table 9.5), substantially reducing population size and, consequently, significantly affecting final heterozygosity (Table 9.5). $r_{(Rec)}$ was also reduced. However, the analysis of these statistics was of little inferential value when the carrying capacity was set to 500 because there were too few data points before $N_{(ext)}$ became stable (Figure 9.1). Despite this limitation, the reduction of $r_{(Rec)}$ was so dramatic that it must be considered biologically meaningful.

Inbreeding, when acting alone, did not modify significantly any population parameters when the populations started from a high level of heterozygosity (80%; Table 9.6). Instead, a significant reduction in $N_{(ext)}$ was evident, when the starting heterozygosity value was low (64%; Table 9.6). None of the scenarios obtained a $P_{(Ext)}$ that raised concerns in the investigated time frame. It has to be stressed, though, that in the long term inbreeding imposed a serious inflection in the demography of the population when the heterozygosity reached values lower than 50%, driving some scenarios to 100% $P_{(Ext)}$ (Figure 9.2).

The additive effect of predation and inbreeding showed dramatic consequences. Even at relatively high starting heterozygosity values, inbreeding in conjunction with predation had a significant effect on $N_{(ext)}$ and subsequently final heterozygosity (Figure 9.2). Depending on the combination of the starting level of genetic diversity, carrying capacity and number of lethal equivalents, inbreeding had a negative, significant influence on $r_{(pop)}$ and increased $P_{(Ext)}$ up to 94% with the projections of the average population size being either reduced by 50% or close to zero in most scenarios with 12 lethal equivalents (Table 9.6; Figure 9.2).

Woylie populations could sustain an additional 18% mortality rate for each age class relative to the baseline model (i.e. an average juvenile and subadult mortality rate of 28%, and 22% for adults per time-unit). Above this limit, the probability of extinction increased with dramatic consequences. For example, with an additional mortality of 19%, the probability of extinction was 90% with an average time to extinction of 64.8 years (SD 1.99).

Table 9.5 Summary of final population parameters and statistical analysis for different scenarios without inbreeding.

Scenario	$N_{(ext)}$ (SD)	$p(N_{(ext)})$	$r_{(Rec)}$ (SD)	% change	$p(r_{(Rec)})$	$r_{(pop)}$	$p(r_{(pop)})$	*Ht* (SD)	p(*Ht*)	$P_{(Ext)}$
Baseline	4959 (480)		0.22 (0.02)			0 (0.06)		0.989 (0.001)		0.00
baseline_K(500)	498 (53)		0.13 (0.11)			0 (0.01)		0.91 (0.021)		0.00
baseline_K(1000)	986 (100)		0.17 (0.09)			0 (0.01)		0.953 (0.008)		0.00
baseline_K(2000)	1988 (215)		0.21 (0.04)			0 (0.01)		0.976 (0.003)		0.00
Ad_Mor(100)	5037 (452)	0.246	0.18 (0.05)	-19.9%	0.007	0 (0.02)	0.969	0.988 (0.001)	0.000	0.00
Ad_Mor(200)	5038 (426)	0.222	0.15 (0.06)	-32.9%	0.002	0 (0.02)	0.878	0.987 (0.001)	0.000	0.00
Ad_Mor(300)	4845 (490)	0.098	0.13 (0.03)	-39.1%	0.001	0 (0.01)	0.986	0.986 (0.001)	0.000	0.00
Ad_Mor(400)	4843 (427)	0.072	0.11 (0.03)	-49.6%	0.001	0 (0.01)	0.992	0.984 (0.001)	0.000	0.00
Ad_Mor(500)	4710 (373)	0.000	0.08 (0.03)	-63.3%	0.001	0 (0.01)	0.992	0.982 (0.002)	0.000	0.00
Juv_Sub_Mor(100)	4975 (468)	0.813	0.18 (0.04)	-20.0%	0.002	0 (0.01)	0.936	0.989 (0.001)	0.120	0.00
Juv_Sub_Mor(200)	4889 (514)	0.324	0.14 (0.03)	-36.0%	0.001	0 (0.01)	0.992	0.989 (0.001)	0.001	0.00
Juv_Sub_Mor(300)	4847 (445)	0.090	0.1 (0.02)	-53.3%	0.001	0 (0.01)	0.935	0.989 (0.001)	0.020	0.00
Juv_Sub_Mor(400)	4722 (447)	0.000	0.05 (0.01)	-75.1%	0.001	0 (0.01)	0.978	0.988 (0.001)	0.000	0.00
Juv_Sub_Mor(500)	20 (12)	0.000	Negative	NA	NA	-0.01 (0.04)	0.124	0.452 (0.225)	0.000	0.93
PY_Mor(100)	4911 (526)	0.501	0.19 (0.06)	-14.3%	0.009	0 (0.01)	0.905	0.989 (0.001)	0.120	0.00
PY_Mor(200)	5024 (508)	0.359	0.19 (0.05)	-14.3%	0.009	0 (0.01)	0.988	0.989 (0.001)	0.410	0.00
PY_Mor(300)	4945 (480)	0.833	0.19 (0.03)	-14.5%	0.013	0 (0.01)	0.914	0.989 (0.001)	0.003	0.00
PY_Mor(400)	5027 (464)	0.315	0.17 (0.06)	-24.9%	0.002	0 (0.02)	0.995	0.989 (0.001)	0.000	0.00
PY_Mor(500)	4928 (491)	0.649	0.17 (0.04)	-25.0%	0.002	0 (0.01)	0.953	0.99 (0.001)	0.000	0.00
%MalesBreed(60)	5059 (472)	0.143	0.2 (0.06)	-8.7%	0.701	0 (0.02)	0.927	0.988 (0.001)	0.000	0.00
%MalesBreed(90)	5038 (462)	0.241	0.22 (0.03)	-1.7%	0.972	0 (0.01)	0.951	0.989 (0.001)	1.000	0.00
%MalesBreed(100)	5025 (541)	0.368	0.19 (0.08)	-14.6%	0.753	0 (0.02)	0.933	0.989 (0.001)	0.435	0.00
InitPopSize(100)	4908 (547)	0.480	0.21 (0.05)	-6.3%	0.279	0 (0.02)	0.950	0.983 (0.001)	0.000	0.00
InitPopSize(500)	5022 (462)	0.350	0.19 (0.07)	-12.2%	0.182	0 (0.01)	0.995	0.99 (0.001)	0.000	0.00
PredNoBait(1)	1794 (47)	0.000	0.06 (0.06)	-72.1%	0.001	0 (0)	0.979	0.961 (0.006)	0.000	0.00
PredNoBait(2)	0 (0)	NA	Negative	NA	NA	NA	NA	0 (0)	NA	1.00
PredYesBait(100)	3575 (630)	0.000	0.12 (0.06)	-46.7%	0.001	0 (0.01)	0.988	0.98 (0.002)	0.000	0.00
PredYesBait(500)	364 (66)	0.000	0.04 (0.03)	-68.6%	0.068	0 (0.01)	0.981	0.839 (0.042)	0.000	0.00

'p(parameter)' = p value of statistical comparison of the population parameter with the baseline model with an equivalent K (e.g. InitPopSize(500) is compared with baseline_K(500)). Note statistically significant values (p<0.05) are presented in bold; '% change' = difference (in percentage) of r(Rec) from the baseline scenario; 'SD' = standard deviation. r(Rec) (only) is over a period of 91 days. Other parameter abbreviations are listed in Table 9.2 and abbreviations for scenarios are listed in Table 9.4.

Table 9.6 Summary of final population parameters and statistical analysis for different scenarios with inbreeding.

Scenario	$N_{(ext)}$ (SD)	p($N_{(ext)}$)	$r_{(Rec)}$ (SD)	% change	p($r_{(Rec)}$)	$r_{(pop)}$	p($r_{(pop)}$)	*Ht* (SD)	p(*Ht*)	$P_{(Ext)}$
Ht: 80%										
baseline	4999 (478)					0 (0.01)		0.792 (0.074)		0.00
baseline_K(500)	489 (44)		0.13 (0.11)			0 (0.01)		0.727 (0.086)		0.00
baseline_K(1000)	1006 (84)		0.17 (0.09)			0 (0.01)		0.77 (0.061)		0.00
baseline_K(2000)	2004 (200)		0.21 (0.04)			0 (0.01)		0.787 (0.073)		0.00
inb3K(500)	501 (50)	0.077	0.17 (0.1)	25.7%	0.593	0 (0.01)	0.908	0.728 (0.089)	0.917	0.00
inb3K(1000)	980 (100)	0.051	0.17 (0.09)	-1%	0.499	0 (0.01)	0.763	0.761 (0.078)	0.372	0.00
inb12K(500)	484 (35)	0.352	0.17 (0.08)	32.9%	1.000	0 (0.01)	0.890	0.746 (0.084)	0.102	0.00
inb12K(1000)	971 (86)	0.004	0.2 (0.06)	16.1%	0.345	0 (0.01)	0.876	0.779 (0.057)	0.282	0.00
inb3_PredK(500)	307 (56)	0.000	0.03 (0.03)	-73.8%	0.068	0 (0.01)	0.959	0.664 (0.115)	0.000	0.00
inb3_PredK(1000)	626 (103)	0.000	0.06 (0.05)	-68.1%	0.043	0 (0.01)	0.732	0.721 (0.113)	0.000	0.00
inb12_PredK(500)	70 (14)	0.000	0.03 (0.03)	-75.1%	0.068	-0.01 (0.01)	0.000	0.587 (0.142)	0.000	0.00
inb12_PredK(1000)	257 (29)	0.000	0.05 (0.05)	-68.4%	0.043	0 (0.01)	0.033	0.702 (0.092)	0.000	0.00
inb12_PredK(2000)	647 (75)	0.000	0.07 (0.06)	-64.5%	0.008	0 (0.01)	0.313	0.758 (0.075)	0.006	0.00
Ht: 64%										
baseline_K(500)	506 (45)					0 (0.02)		0.589 (0.154)		0.00
inb3K(500)	493 (41)	0.032				0 (0.02)	0.655	0.592 (0.144)	0.865	0.00
inb12K(500)	462 (40)	0.000				0 (0.01)	0.732	0.605 (0.161)	0.475	0.00
inb3_PredK(500)	272 (38)	0.000				0 (0.01)	0.646	0.554 (0.147)	0.105	0.00
inb3_PredK(1000)	577 (93)	0.000*				0 (0.01)	0.906*	0.583 (0.141)	NA	0.00
inb12_PredK(500)	9 (4)	0.000				-0.03 (0.04)	0.000	0.432 (0.174)	0.000	0.94
inb12_PredK(1000)	38 (17)	0.000*				-0.02 (0.01)	0.000*	0.475 (0.185)	NA	0.03
inb12_PredK(2000)	198 (36)	0.000*				-0.01 (0)	0.003*	0.559 (0.163)	NA	0.00

'p(parameter)' = p value of statistical comparison of the population parameter with the baseline of equivalent K (e.g. *InitPopSize(500)* is compared with *baseline_K(500)*). Note statistically significant values (p<0.05) are presented in bold; ' % change' = difference (in percentage) of $r_{(Rec)}$ from the baseline scenario; SD = standard deviation. Other parameter abbreviations are listed in Table 9.2 and abbreviations for scenarios are listed in Table 9.4. *Statistical tests carried out with baseline scenario not realigned to heterozygosity starting value of 64% for prohibitive computational times. $r_{(Rec)}$ (only) is over a period of 91 days.

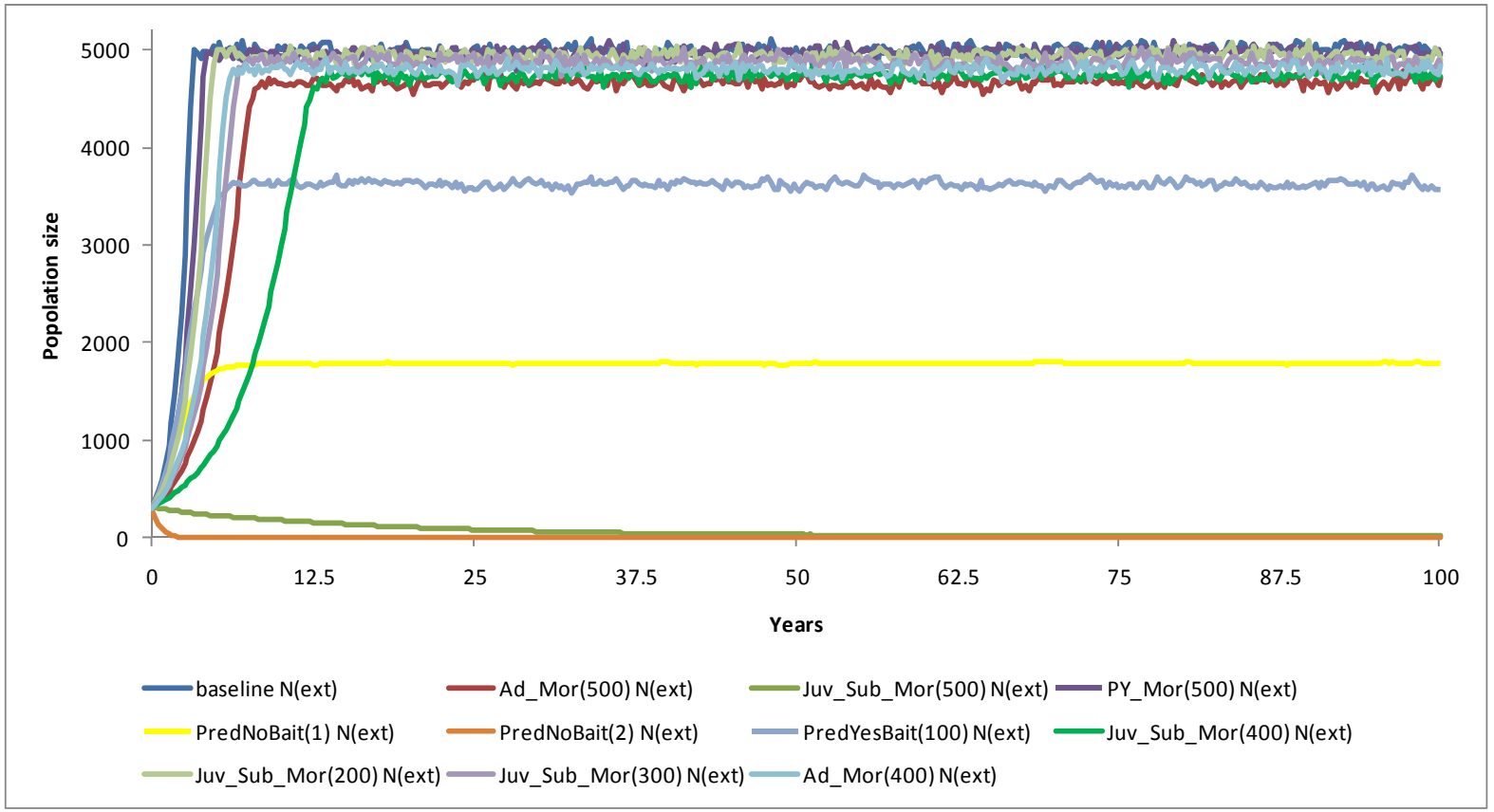

Figure 9.1 Representative curves showing predicted average population sizes for selected scenarios.

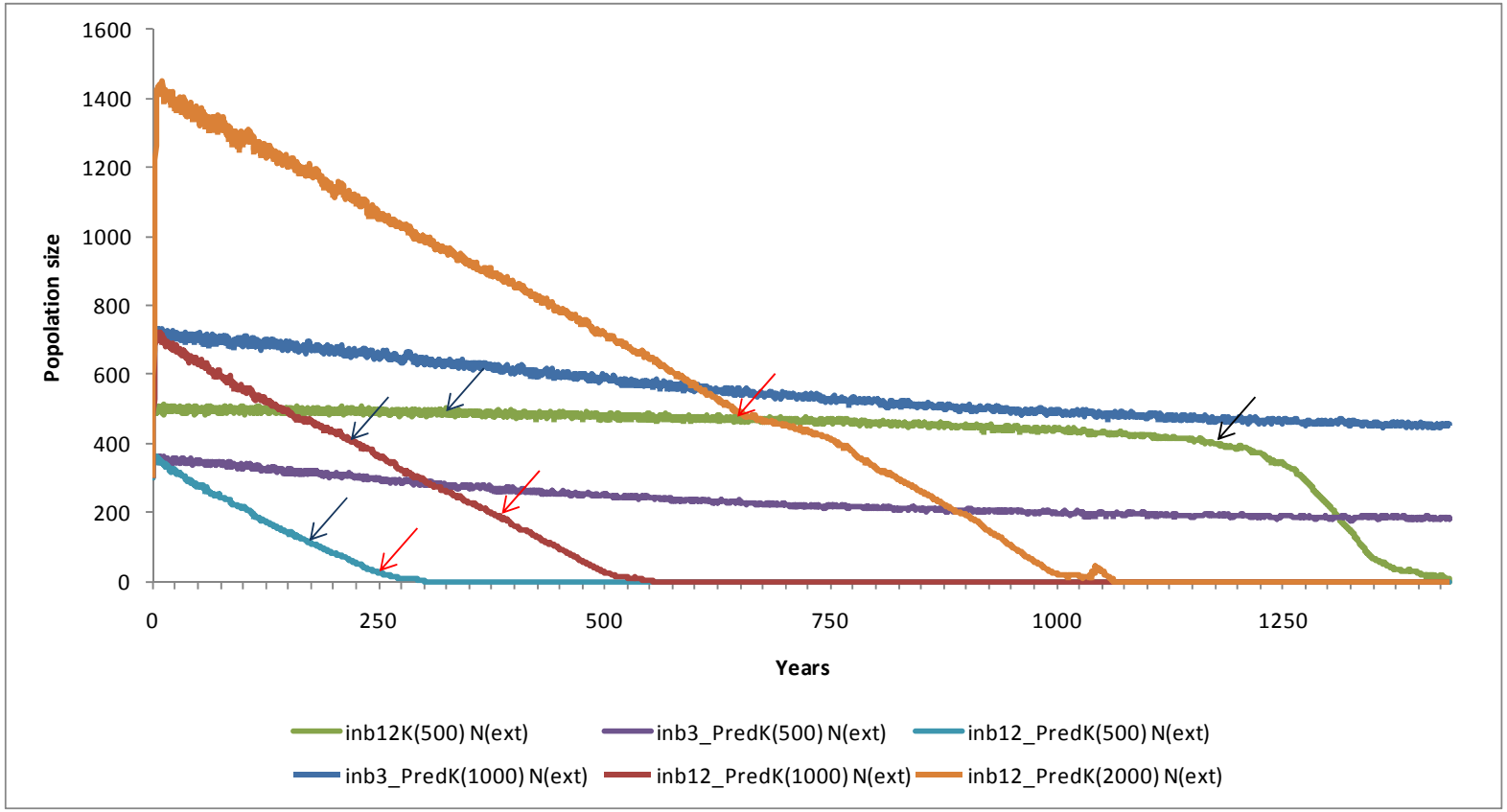

Figure 9.2 Representative curves showing predicted average population sizes for selected scenarios with inbreeding included in the simulation.

Black arrow indicates average expected heterozygosity (Ht) of 47%; red arrows, Ht = 64%; blue arrows, Ht = 80%.

9.3.3 Model validation

Model validation is a critical step in order to make PVA a valid and reliable tool in wildlife research and management. The four criteria recommended for acceptance of models were scrupulously evaluated (Bart 1995).

1. Structure of the model: The chosen model accommodates both biological and ecological features in woylies. Assumptions were critically evaluated and none were judged to seriously invalidate the outcomes of the model. Although some limitations posed by these assumptions were recognised, these are expected to bias the analysis only in a minimal and predictable way.

2. Parameter values: Most values used were reported in more than one study providing reasonable confidence in their reliability and most of them were, at least broadly, confirmed by more recent field work (DEC Science Division 2008a). Additionally, thorough sensitivity testing was carried out, as advised, and precautions were taken accordingly in the interpretation of the results.

3. Secondary predictions of the model: Secondary predictions (Bart 1995), such as mortality rates for age groups indirectly derived by the model, were compared to the ones of the sister taxon boodie (*Bettongia lesueur*) and were found to be consistent. The use of sister taxa for gathering biological and demographic information has been suggested and successfully used when such data were not available for the species of interest (Lindenmayer et al. 1993a; Bart 1995; Heinsohn et al. 2004). Under the same rationale, a comparison with boodies was considered appropriate to validate the model in light of the strong similarity in their physiology

and ecology (Hinds & Smith 1992; Robley 1999; Short & Turner 1999; Johnson & Delean 2001; Parsons et al. 2002; Pizzuto et al. 2007).

The subadult mortality rates used for woylies were comparable to those calculated in boodies, providing further support for the reliability of these values. Furthermore, as expected the maximum exponential growth rate calculated in woylies (and used in the baseline scenario, 0.92) was higher than that obtained in the boodie populations on Dorre Island (0.75, Short & Turner 1999). A lower island population maximum growth rate was expected because of the demonstrated seasonal fluctuations in the reproduction success (Short & Turner 1999), which were not observed in any of the woylie populations (Sampson 1971; Christensen 1980; Ward et al. 2008a) for which this model was intended to be used. Also the island may be less productive than the more mesic forests and woodland systems where the extant woylie populations persist in south west of Western Australia.

The deterministic calculations of the generation time obtained from the model (i.e. without considering stochastic variation: females = 2.43 years; males = 2.65) are consistent with the generation time calculated from trapping data in Upper Warren (Pacioni and Wayne, unpublished data) and for the average of the species (< 3 years, Groom 2010).

4. Primary predictions of the model: The model was tested under different conditions and several expectations were met by the outcomes of the simulation analysis. The phase of exponential growth was evident only in the first few years (3-4) of the simulations, similar to that actually observed in Upper Warren (Table 9.3; Figure 9.1), Batalling and Dryandra (Orell 2004) after the beginning or intensification of baiting programs.

In the scenario where predation was modelled without fox baiting, $N_{(ext)}$ was reduced to a similar value to what can be estimated for the wild populations from trapping data before the beginning of any baiting (Christensen 1980) and there was no population extinction in the modelling as supported by field data. When the density dependency was removed from the function (see above) the populations became extinct (Figure 9.1), as happened at other sites that did not contain *Gastrolobium* vegetation (e.g. Priddel & Wheeler 2004; Martin et al. 2006).

The $r_{(Rec)}$ calculated in the scenarios with both predation and a baiting program (*PredYesBait(100)*) was similar (i.e. no significant difference, p=0.941) to the average growth rate calculated from field data in the presence of predation and fox baiting (Table 9.3).

Little difference in $r_{(Rec)}$ was expected from the alteration of pouch young mortality rates due to the embryonic diapause (Tyndale-Biscoe 1984) and the short birth interval (Sampson 1971; Christensen 1980) as was highlighted during the sensitivity testing.

9.4 Discussion

The model used in this study fulfilled the four criteria stated by Bart (1995) to be considered a reliable technique of investigation and inference on population general trends over time.

The values of carrying capacity investigated (500, 1000 and 2000) were chosen because the $N_{(ext)}$ obtained were similar to current woylie population size estimations (Groom 2010) and starting values of genetic diversity (64 and 80%) were similar to that found in wild woylie populations (Chapter 6). Consequently, trends evident in the analysis would be expected to be

of relevance for these populations. Inbreeding alone appeared to be of little concern for the projections of populations with 500 or more individuals. Nevertheless, in the long term (~600 years when the starting Ht = 64%, Scenario *Inb12K(500)*, Figure 9.2) it was demonstrated to have a substantial impact on population demography.

Perhaps the most striking result of this PVA is the evidence that the main threatening process is a result of the interaction of various variables (particularly predation and inbreeding) that acquired a considerable strength together, whilst not being greatly significant by themselves. A disease with mild or no clinical consequences may cause a reduction of the animal fitness similar to that which was modelled for the inbreeding depression. Consequently, by extension, the demonstration of the interaction between predation and inbreeding depression provides evidence that the interaction between predation and diseases could pose a serious threat to the population persistence, even when the disease does not cause a severe clinical condition.

The PVA results suggest that populations at current sizes (300-500 individuals) are definitely at risk especially when considered that the model probably overestimates the genetic diversity (see Section 9.2.1) and the fact that other, potentially important, stochastic events were not included in the model (e.g. bush fires). More than twice the current population size should be targeted as a minimum size (i.e. >1000-2000 individuals) to ensure woylie conservation. This is particularly true for the population in Tutanning Nature Reserve. This population (N=300), in the model projections, reached heterozygosity values close to the threshold (~50%) where inbreeding depression, if present, would cause a serious inflection in the population demography or even local extinction if not otherwise mitigated.

Population genetic theory predicts that an effective population size of 50 for short term and 500 for long term conservation should be targeted (Franklin 1980; Franklin & Frankham 1998),

which would indicate that population sizes should be above 500 for short term and 5000 for long term conservation (Frankham 1995a). These expectations are broadly supported by theoretical simulations and empirical data on several vertebrates (Thomas 1990; Nunney & Campbell 1993) including macropod species such as euros (*Macropus robustus isabellinus*) (Short & Turner 1991). The PVA estimates are consistent with these theoretical and empirical expectations.

The models also provided an approximate estimate of the maximum mortality rates that can be sustained by woylie populations (i.e. an average juvenile and subadult mortality rate of 28%, and 22% for adults per time-unit). These values could have several applications in terms of species management and conservation. Namely, it can set a minimum threshold by which feral predator control needs to be effective. It provides an indication of the level of mortality that would cause a pattern of decline similar to that observed. In this matter, it would be possible to investigate whether the decline was the result of predation alone if further field studies could quantify the mortality caused by feral predation.

The process of developing a model is often recognised as an important step to understand the dynamics of a population decline. This is achieved by promoting the analysis of biological and ecological features that may influence population dynamics and is, therefore, considered one of the main achievements of PVA (Lacy 1993; Lindenmayer et al. 1993a; Starfield 1997; Morris & Doak 2002). In this study, the limited information on mortality rates especially with regards to juvenile and subadult groups was one of the major challenges of the PVA. Due to behavioural differences (i.e. lower trappability) there is a lack of information on the juvenile and subadult age groups (A. Wayne, personal communication). This highlighted the importance of improving the knowledge on this key population parameter, which requires a specific monitoring effort for this age class. On this matter, it is of interest to note that in the

forest blocks where the woylie decline had already occurred or was occurring (Balban, Boyicup and Winnejup) none or very few subadults were caught in contrast with blocks where the population density was still reasonably high (Ward et al. 2008a). The critical demographic role of this age group, as demonstrated in the model, indicates that an increased juvenile and subadult mortality rate could possibly explain the recent steep decline in the Western Australian woylie populations.

The process of building a model also emphasized the lack of information on the mortality rate due to fox predation and the unavailability of reliable methods to quantify the efficiency of the baiting program.

9.4.1 Limitations of the model

Models are necessarily a simplification of real life, hence their results are an approximation of what would happen if the conditions described in the model are met and should be interpreted carefully (Starfield 1997).

Mortality rates and predation from feral animals were extrapolated from available data and the uncertainty that surrounds these estimates poses a risk for the reliability of the model. Future studies that would more rigorously assess these variables could further improve the reliability of the model.

Growth rates (as well as the other population parameters) are not a species characteristic but rather a species-environment interaction attribute (Caughley 1971; Caughley 1977). This model should be considered a good approximation of woylie population dynamics, as long as

the environmental conditions remain similar to the condition in which the parameters were obtained. It can be assumed that the results from the model presented here can provide a reliable indication of possible projections for woylie populations in the southwest and wheatbelt regions (Batalling, Dryandra and Tutanning) because it appears that there is a strong similarity between these populations and the populations in Upper Warren (from which fertility and mortality rates were estimated). Nevertheless, this model should be adjusted whenever there is evidence of different environmental conditions to those present in Upper Warren. For example, in case the model was used for the population in Karakamia, it should be adjusted for the different breeding patterns (seasonal versus year-round). Similarly, the effect of feral predation was modelled with a density dependent function, but this may be site specific (e.g. due to different vegetation structure, carrying capacities, site productivity levels, etc). In such a situation, the absence of a baiting program would drive the population to extinction (e.g. Priddel & Wheeler 2004; Martin et al. 2006). The model seemed to be easily adaptable to this situation, as shown by the scenario *PredNoBait(2)*, where the effect of predation is not modelled with a density dependent function; however proper validation is needed.

Lastly, other stochastic sources of variation, such as additional mortality caused by bush fires and reduction of resources during droughts, could be included in the model to obtain more conservative outcome predictions of different management strategies.

9.5 Conclusions

A population viability model was successfully created and, with the due considerations in regard to the limitations discussed above, the proposed aims were achieved. It was possible to identify population parameter thresholds and disclose the power of interactions between factors.

Fundamental information for the conservation of the woylie was acquired. A minimum population size of 1000 individuals should be targeted for conservation purposes and juvenile and adult mortality rates should be kept below 28% and 22% per time-unit, respectively, in order to guarantee a positive population growth. These mortality rates represent a minimum level of effectiveness that should be achieved through feral predator control programs. Additionally, if a quantification of the mortality caused by feral predators becomes available, the model would provide a theoretical framework to further test the suggested hypothesis of the role of feral predation as a cause of decline in woylie populations (Start et al. 1995) as well as other Australian marsupials (Burbidge & McKenzie 1989; Short & Smith 1994; Cardillo & Bromham 2001).

Given the critical nature of the tasks that wildlife managers are called to carry out, it is important to interpret the results from PVA cautiously. A multiplication of the modelled minimum population size by a factor of eight has been suggested as a conservative approach to take into account for the effects of unknown variables, measurement and encoding errors and possible inconsistencies between management recommendations and their practical execution *"before one would be satisfied that an adequate buffer against misfortune had been built into recommendation."* (Caughley & Gunn 1996, p. 206).

Genetic diversity seems not to be an issue at the moment; however, given the recent substantial declines in populations to typically less than 500 individuals each, it is possible that disadvantageous alleles will accumulate, decreasing the population fitness and their evolutionary potential (Frankham et al. 1999), as well as possibly increasing disease susceptibility (Little & Ebert 2001; Charpentier et al. 2008; Acevedo-Whitehouse et al. 2009). Additionally, the PVA demonstrated the synergistic effects of inbreeding and feral predation, indicating that the potential increase of inbreeding should be reason for concern.

Developing the model highlighted the areas where more research is needed (e.g. mortality rates in juveniles and subadults, predation rates, etc). Moreover, the model will serve as a basis for future developments to investigate threatening processes and management strategy outcomes in woylie populations, and, by comparison, other endangered species with a similar ecology and living in similar ecosystem.

10 General discussion

The broad aim of this research project was to contribute to the knowledge on the general health and ecological attributes of woylie populations that were considered directly relevant for the conservation and recovery of the species in the context of the recent and dramatic population decline.

Various health and ecological aspects were considered and, when appropriate, results from one component of the research were used to inform and guide the analysis of other components. For example, genetic analyses were used to provide the genetic parameters in the PVA or to define groups (i.e. populations) for haematological comparisons. On several occasions the results from the different aspects were mutually supportive, such as the evidence of genetic and haematological differences between populations or the consistent estimation of the minimum viable population size based on the genetic analysis and PVA.

In this chapter, overall achievements and the collective strength of the findings of this study are synthesised and discussed within the framework of four main areas: health status of woylie populations; woylie ecology; woylie recovery; and woylie conservation and management. In light of the collaborative nature of the WCRP, expected contributions from this research to other components of the WCRP (e.g. influence of climate and resources on woylie population dynamics; significance of trypanosomes and piroplasms infection, etc.), as well as future recommended studies, are also outlined.

10.1 Health status of woylie populations

Disease can be defined as "any impairment that interferes with or modifies the performance of normal function" (Wobeser 1997 p.1). This definition particularly suits wildlife research where the interest is more on the population level rather than on the individual clinical condition. In this context, disease investigation in wildlife is notoriously difficult for several reasons. Comprehensive knowledge about potential causes that could be responsible for reduced fitness of a population is seldom available (Wobeser 2007). Additionally, pathogens can act on a population level in very subtle ways and can affect population dynamics not only by increasing mortality rates, but also through sublethal manners, such as affecting reproductive success. For example, Cowpox virus infection in bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*) is asymptomatic (Bennett et al. 1997; Feore et al. 1997), and does not affect the mortality rate of these hosts (Bennett et al. 1997). Nevertheless, it is capable of altering population dynamics of the hosts by delaying their first litter (Feore et al. 1997). An experimental study demonstrated that the energy cost of an immune response to non-pathogenic antigens alone may be responsible for reduced survival (Hanssen et al. 2004). These examples illustrate how subtle the regulating mechanism of a pathogen can be and consequently, how difficult it is to provide substantial and scientifically sound evidence of the demographic limitations imposed by the presence of a particular aetiological agent in a population.

Various factors that could potentially affect the health of woylie populations were considered in this study, spanning from individual and population genetic profiles to the presence of selected pathogens. General health indicators, such as haematology and physical condition, were also considered.

The WCRP attempted to recover woylie carcasses to assess causes of mortality and obtain an indication of whether pathological processes were involved in the recent declines, but unfortunately, only two of 21 collared woylies that died were suitable for post mortem examinations, since all the others had been either predated or scavenged, or were in an advance state of decomposition when located (Ward et al. 2008b).

Sampling wild animals that are showing clinical signs or retrieving carcasses to use for pathological examination can be extremely difficult. This was also shown in an experimental study, where only 12% of the marked carcasses placed in "exposed position atop vegetation" in wetland habitat were retrieved and none of the ones placed in low visibility locations were found by a search crew (Stutzenbacher et al. 1986). Other studies have demonstrated that the recovery rate is variable but always extremely low, with an average recovery rate of 7% (Wobeser 2007).

It is possible that a pathogen may not be responsible for obvious clinical signs and still affect woylies in a way that could make them more susceptible to predation. Detection of such a disease would be extremely challenging.

Due to the lack of direct evidence of disease from field data, a qualitative approach was used to prioritise investigations into selected pathogens as suggested by others (Armstrong et al. 2003; Miller 2007; Watson et al. 2007). Several pathogens were identified as a high priority for further investigation including, but not limited to, Macropod Herpesvirus (MaHV), Macropod Orbivirus, and Encephalomyocarditis virus (Chapter 2).

The results of the analysis of the body condition indices and haematological parameters are supportive of the general hypothesis that if a disease is responsible for, or a co-factor of, the

recent woylie decline, it is unlikely to be a chronic condition (Wayne 2006; Thompson et al. 2008; Chapter 2), which would be expected to result in changes to the parameters measured. Additionally, the virological investigations did not detect any immune response to viruses known to occur in the area (Chapter 4), while the probability of detecting seropositive animals is expected to increase if the condition caused by the agent is chronic (Thrusfield 2007).

There is increasing evidence that genetic attributes may be key factors in the decline of certain threatened species. Low levels of genetic diversity in the Tasmanian devil (*Sarcophilus laniarius*), at both microsatellite and major histocompatibility complex (MHC) loci, was recognised as the primary cause of increased susceptibility to the transmission of a fatal clonal tumour, which ultimately has been responsible for the dramatic decline of this species (Jones et al. 2004; Siddle et al. 2007). Other examples of the impact of low genetic diversity on species, include increased extinction probability in some butterfly species (Saccheri et al. 1998), population declines in the greater Prairie chicken (*Tympanuchus cupido pinnatus*) (Westemeier et al. 1998) and the adder (*Vipera berus*) (Madsen et al. 1996), and reduced survival in the golden lion tamarin (*Leontopithecus rosalia rosalia*) (Dietz et al. 2000). These considerations, associated with the PVA projections for woylies (Chapter 9), suggest that proper genetic monitoring and management is warranted in order to guarantee future genetic viability of this species.

Genetic diversity in woylie populations is generally relatively high and it does not appear to be a contributing factor to the recent decline (Chapters 6 and 7). However, particular concern was raised that among the indigenous populations, woylies in Tutanning have a substantially reduced genetic diversity (H_E = 0.64) that may detrimentally manifest itself either currently or in the future if not adequately addressed (Chapter 6). Among the translocated populations the

populations on the South Australian islands also had substantially lower levels of genetic diversity (Chapter 7).

Even though none of the factors evaluated in this study could be clearly linked with the woylie decline, the evidence of different haematological responses between populations and the high prevalence of health problems detected during field health examinations (Chapter 3) may indicate that disease could have played a role in the decline. The collective strength and weight of the evidence to date, although incomplete, is therefore, sufficient to merit further enquiry of the possible role of disease in the recent woylie declines (see Section 10.5).

In summary, this research project has greatly improved the available knowledge on the health and viability of woylie populations. Haematological and serological information represent baseline data that will enable monitoring and detection of changes in the health status and contribute to the management of the species (see Section 10.4). This study also provided the first quantification and definition of genetic "health" in the woylie (see Section 10.4) and identified populations at risk of reduced genetic fitness (i.e. populations in Tutanning and South Australian islands). Additionally, these results will contribute to and facilitate ongoing research in other components of the WCRP. For example, haematological profiles can be used to better understand physiological effects of the different diets (Rodda et al. 2008) and climate conditions (Orell 2008; see also Chapter 3 for further details). Similarly, haematology and genetics can be used to improve the understanding of the role of pathogens currently under investigation such as trypanosomes (Smith & Averis 2008; Smith et al. 2008a), piroplasms (Clark & Spencer 2007) and gastro-intestinal parasites (Parker et al. 2008) (see Section 10.5 for details).

10.2 Woylie ecology

Important insights were gained into woylie population structure and dynamics. For example, woylie female philopatry was confirmed and the genetic consequences of this behaviour were described (Chapter 8). The lack of differences in body condition indeces and haematological parameters between the two genders in woylies from Karakamia (Chapter 3) reinforces the hypothesis that the population in Karakamia is a high density population with limited resources. The morphological and physiological adaptability of this species is further demonstrated by the different breeding modality in this population (i.e. seasonal breeding as opposed to continuous breeding) without necessarily implying a substantial modification of the genetic profile, as the Bayesian analysis assigned individuals from Karakamia and Dryandra to the same cluster (Chapter 7). Similar flexibility has also been shown by other Australian marsupial species. For example, morphometric modifications in the common brushtail possum were evident in introduced individuals in New Zealand in less than 35 generations (Yom-Tov et al. 1986), without a significant difference in the overall genetic profiles of these populations (Taylor et al. 2004).

In the Upper Warren region, it is now evident that two populations exist where woylies were previously considered and managed as one. The Kingston and Perup populations were haematologically (Chapter 3) and genetically (Chapter 6) distinct, suggesting that there are not only physical barriers (Perup River), but also environmental differences that contribute to the differentiation of these two populations. This hypothesis is also supported by dietary differences between the two areas (K. Zosky, personal communication). Additionally, marginal genetic divergence between the two Upper Warren compartments (Kingston and Perup) was

found in the genetic structure of a sympatric species, the chuditch (Western quoll, *Dasyurus geoffroii*. F_{ST} = 0.043, p = 0.001) (M. Cardoso[8], unpublished data).

The evidence of gene flow within each population (i.e. up to 40 km from north to south, Chapter 6) is not only relevant for the conservation management of woylies, but also for the risk assessment of directly transmitted diseases. It is plausible that direct transmission of an aetiological agent could happen throughout each population within the time frame experienced in the recent decline. In this scenario, the propagation of the infection is most likely sustained by a "neighbour to neighbour" mechanism given the spatial genetic gradient (i.e. isolation by distance, Chapter 8), which is consistent with the observed spatial pattern of the decline (Wayne 2006; Wayne et al. 2008b). Additionally, it is evident that, even though woylies generally disperse short distances, long distance movements do occur at a low rate (Chapter 8). This finding, coupled with the proven dispersal between Kingston and Perup (2-3% dispersal rate, Chapter 6), provides evidence that direct transmission of disease agents from one compartment of the Upper Warren region to the other is also possible. Furthermore, the genetic analysis provided evidence that woylie populations in Dryandra and Upper Warren are not completely isolated (Chapter 8). Therefore, disease transmission between these populations is also possible.

Furthermore, woylie genetic molecular data should be considered when assessing the likelihood of naturally occurring epidemics or a human-mediated alteration of the transmission mechanism of the infection, if a directly transmitted disease is confirmed as the cause of the recent decline. Bayesian assignment analyses identified, under the most flexible model, that the majority of dispersal events (75%, n=6) occurred in the north-easterly compartment of the Upper Warren region (Appendix 3-4) and it was speculated that the Perup River and associated

[8] Cardoso, M. J. unpublished. Conservation Genetics of Australian quolls (Dasyuridae: Marsupialia), PhD thesis. University of New South Wales.

agricultural land represents the physical barrier responsible for the differentiation of the two populations in Upper Warren (Chapter 6, Figure 6.2). Consequently, based on the available data, the north-central and eastern forest blocks (Corbal, Balban and Keninup) represent the greatest connection between Kingston and Perup populations. Problems associated with the discriminatory power of the genetic data are the small sample sizes obtained from the southern blocks (Boyicup and Chariup – due to the advanced stage of decline of these sites), which possibly reduced the detection of migrants across the southern interface of the two populations (boundary between Warrup and Boyicup) and the assumption that dispersal dynamics pre-decline (i.e. high density populations) were the same as those detected in this study with most forest blocks at low density. With due consideration of these limitations, a naturally occurring epidemic of a directly transmitted disease could be expected to closely follow the movements of woylies outlined by the genetic analysis.

This research also sets the basis for an adequate sampling design to source animals to establish new translocated populations, augment genetic diversity of existing populations or to create an insurance captive breeding population. For these purposes, animals should be trapped at a distance of at least 1-3 km in order to maximise the probability that individuals are unrelated (Chapter 8). Consideration of the spatial organization of woylie populations is also important when sampling for monitoring of demographic parameters (e.g. fecundity) and disease investigations. An intensive sampling in a limited area (i.e. < 1 km diameter) would actually include family groups rather than individuals representative of the whole population (Chapter 8).

The mtDNA analysis highlighted historical connections between indigenous populations (Chapter 6), which, as a result of the habitat fragmentation caused by humans, are not present anymore. The disruption of this connectivity makes it clear that, despite the relatively large

areas protected for conservation in Western Australia, these regions only represent patchy and fragmented remnant habitat, as compared to the complex ecosystem that was once present before European settlement. Consequently, the natural dynamics that used to occur in the past are currently limited, possibly increasing vulnerability to stochastic events and genetic drift (Saccheri et al. 1998; Frankham et al. 2002; Fischer & Lindenmayer 2007).

10.3 Woylie recovery

Potentially, woylie populations could suffer only limited genetic "damage" if there is no delay in the identification and removal of the cause of the decline. PVA projections (Chapter 9) indicated that woylie populations can recover quickly (within 3-4 years) when they are released from limiting factors (given that resources are sufficient) and that the loss of genetic diversity, during this process is limited (~ 1%, Table 9.2, baseline model). The large "intrinsic" growth rate of woylies was empirically proven by the substantial increase in abundance after the establishment of the baiting programs (Orell 2004). These considerations highlight the conservation value of the efforts put in place to identify the cause of the recent decline.

The PVA quantified the mortality rates that would be required to have a decline at the rate experienced (i.e. more than an average of 28% for juvenile and subadults and more than 22% for adults per 91 day time period). This means that the cause(s) of the recent decline had to be responsible for considerable and sustained mortality rates. The combination of the results from the spatial analysis (Chapter 8) and PVA (Chapter 9) demonstrated that the subadult cohorts are potentially critically important for the demographic growth and prospects of recolonisation. Therefore, individuals of this age class may represent a vital component in the recovery of woylies. Lastly, the PVA highlighted the potential synergistic effect between feral

predation and inbreeding. By extension, any factor responsible for a reduction of individual fitness, such as disease, may interact significantly with predation and substantially limit the demographic growth or even be responsible for significant population decline.

10.4 Species conservation and management

The significance of the information acquired from this research project is not only restricted to increasing the general knowledge about the species' biology and ecology, but also is directly applicable to the conservation and management of woylies. For example, the information on the genetic diversity of the indigenous populations provides a quantified threshold of genetic diversity (i.e. >70%) that should be considered as the minimum conservation target for both indigenous and translocated populations (Chapter 6 and 7). Due to their geographic isolation, the South Australian islands potentially represent insurance populations. However, their genetic viability is compromised by significant genetic loss. Active management should be undertaken in order to genetically "rescue" these populations, with supplementations probably being the most suitable option in order to achieve this (see Chapter 7 for details). Exploration of different methodologies through PVA and experimental trials in a preliminary stage would certainly maximise the results of this management option.

Active management of the population in Karakamia is also recommended (Chapter 7). This population is representative of Dryandra's genetic stock (Chapter 7) and should be managed as an insurance population. The limited carrying capacity of the sanctuary requires introduction of new animals to limit divergence between the source and insurance populations. When the population reaches an unsustainable density level, a certain number of woylies are trapped and removed (Mawson 2004; Chapter 1). Once again PVA analysis may provide useful

information to quantify the optimal balance of animals to introduce and remove to retain the maximum genetic diversity and limit divergence. For any operation that involves animal movements, quarantine protocols, associated with a thorough disease risk assessment and health screening, should be implemented to reduce the risk of introducing pathogens into naïve populations (Chapter 4).

In order to monitor the outcomes from the above suggested management actions and to correctly implement adaptive management, regular genetic monitoring is necessary. Health monitoring is equally important to maximise the opportunities to detect changes in the health status of woylie populations.

The evaluation of the genetic viability of the translocated populations suggested that the minimum viable population size to maintain an adequate level of genetic variability is between 2,000-3,000 individuals (Chapter 7). Similarly, PVA projections indicated a minimum viable population size of 1,000 (Chapter 9). Introduced predators have for a long time been identified as a key threatening process to woylie populations (Christensen 1980; Burbidge & McKenzie 1989; Start et al. 1998). Adequate feral predator control should be implemented in order to maintain the average mortality rate below levels that would compromise demographic stability or growth (i.e. limit mortality rate of juveniles and subadults to below 28% and adults to 22%, Chapter 9). As a consequence of the inherent inability of satisfactorily predicting stochastic events and incomplete knowledge on important factors that may affect population size a conservative approach should be adopted when using PVA estimates (e.g. using the eight times multiplication factor when establishing the minimum viable population size, Caughley & Gunn 1996). On this basis, woylie populations greater than 8,000 individuals should be targeted to maximise the likelihood of achieving sustainable populations. The importance of this conservative approach is strikingly highlighted by the magnitude and rate of

the current woylie declines that have resulted in population reductions to dangerously low levels where the likelihood of local extinction is now a real possibility (Groom 2010).

Site selection for future translocations and establishment of new populations should favour locations where the minimum viable population size could be expected to be achieved and sustained over time. Additionally, future translocation programs should also carefully design the trapping regime to source founders (see Section 10.2, Chapters 7 and 8), ensure post-establishment growth (e.g. implementing feral predator control programs) and monitor demographic and genetic results in order to appropriately apply corrective measures (i.e. adaptive management).

Knowledge gained through this study about the spatial organisation of woylie populations also suggests that a minimum level of inter-connectivity (i.e. 2%, Chapter 6) between populations in the Upper Warren region should be maintained and this may be achieved by promoting habitat quality (e.g. monitoring the effect of land use for livestock, agriculture and timber harvesting), increasing habitat connectivity (e.g. corridors and ecosystem restoration) and maintaining effective feral predator control.

The gene flow between Upper Warren populations, together with the historical gene flow that was demonstrated between the indigenous populations (see Section 10.2), are additional evidence of the potential benefits that would be obtained by increasing the connectivity between protected areas.

The benefits that can be generally expected by the implementation of corridors in wildlife conservation include the reduction of genetic drift, demographic stochasticity and extinction rates (Wilson & Willis 1975; Harris 1985). The use of wildlife corridors is not free from potential

disadvantages. These are mainly related to the increased risk of spreading diseases, fire, feral predators or other pests and high costs for an uncertain success (Simberloff & Cox 1987; Simberloff et al. 1992; Rosenberg et al. 1997). Nevertheless, based on the increasing evidence of the advantages provided by corridors for both flora and fauna (Lindenmayer et al. 2000; Lindenmayer & Hobbs 2004; Lindenmayer et al. 2006; Jonson 2010), ambitious projects are progressing in different parts of the world, such as the Yellowstone to Yukon conservation initiative (Yellowstone to Yukon Conservation Initiative 2006) or the connection of isolated Iberian lynx (*Lynx pardinus*) populations in the Iberian Peninsula (Delibes et al. 2000).

The southern region of Western Australia (including the southwest and south east coastal areas) is part of a landscape restoration project, called the "Gondwana Link Project" which intends to preserve the habitat of the south-west of Western Australia that is still in its natural state and restore continuous habitat between the Fitzgerald River and the Stirling Range National Parks (Bradby 2008). While it is beyond the scope of this research study to discuss the overall merit of such a large scale project, it is now clear that, despite their small home range and usually short movements, the woylie will probably benefit from a large increase in habitat connectivity in this region.

The conservation of the existing continuous jarrah forests and the establishment of continuous habitat in the southeast coastal areas will not be sufficient, to promote woylie colonisation of new areas and gene flow between populations, by itself. As mentioned previously, woylie numbers will increase only when other factors limiting their growth are controlled throughout the whole system (e.g. feral predators). It is also anticipated that long distance gene flow will not occur in a short time frame (Chapters 6 and 8), but if it does happen, this study will provide the baseline data to quantify possible genetic diversity changes in woylie populations. Additionally, this study provided valuable tools to measure potential risks and benefits. For

example, the PVA model developed here may be used to better quantify the risks associated with infectious diseases. Changes in pasture distribution is expected to produce changes in density and distribution of kangaroos (Johnson & Bayliss 1981), which are potential carriers of MaHV. Kangaroos would normally stay on the edge of the forest in close proximity to pastures (Johnson & Bayliss 1981), but restoration of the land from farming to native flora is likely to alter their distribution potentially increasing the contact rate with woylies. To explore possible consequences of increased contact rates with potential carriers of MaHV (e.g. kangaroos) in the future, it is possible to link the PVA model with OUTBREAK (Pollak et al. 2003) and explore possible population dynamics under different scenarios. The virological investigation carried out could be used as an estimation of the susceptible proportion of the woylie population (i.e. >90%, Chapter 4) to this virus. Similarly, virological investigations may provide indirect estimates of exposure to biological vectors (see Section 10.5) and these estimates can be used to improve the risk assessment for Orbiviruses. On numerous occasions PVA has been used to evaluate changes in the risk of infection under different levels of connections between populations (e.g. Haydon et al. 2002), as well as to predict patch use (e.g. Kramer-Schadt et al. 2005). In the bush rat (*Rattus fuscipes*), agile antechinus (*Antechinus agilis*) and several arboreal marsupials, PVA proved to accurately predict patch occupancy (Lindenmayer et al. 1993b; Lindenmayer & Lacy 2002), highlighting the importance of these preliminary estimations and the reliability of this analytical method.

Where the implementation of natural corridors is hampered by anthropological land use and development, “human-assisted” gene flow through translocations may be considered, especially for populations such as Tutanning where a significant reduction of genetic diversity is evident. These practises were effective in increasing genetic diversity and reducing inbreeding depression in wild populations, for example, in adders (Madsen et al. 1996) or desert topminnow fish (*Poeciliopsis monacha*) (Vrijenhoek 1994). However, a clear

identification of the causes that are currently limiting the population growth (Fischer & Lindenmayer 2000), a careful assessment of the risks (e.g. Cunningham 1996; Jakob-Hoff et al. 2001; Mathews et al. 2006) and a careful evaluation of the genetic profiles of the animals translocated (Goossens et al. 2002; Sigg 2006; Armstrong & Seddon 2008; Williams & Hoffman 2009) is critical prior to the implementation of these management actions.

10.5 Future research

10.5.1 Epidemiology

No unequivocal evidence supporting an association between changes in the health status of woylies and the recent decline was found. On the other hand, the evidence collected to date indicates that disease cannot be dismissed as a possible cause or co-factor of the decline (Chapters 3 and 4), especially in combination with other factors such as predation. Future work that is recommended to better define the potential role of a reduction of fitness in the woylie decline would include the completion of the epidemiological analysis of available data (see Chapter 3 for details) and through inclusion of data currently being collected by other researchers on health parameters, pathogens and possible secondary determinants, such as environmental or genetic variables. Potential cryptic and synergist effects should also be investigated. These analyses should be linked with fecundity and survival data (when available), to facilitate a broad and correct interpretation of the role of aetiological agents.

A longitudinal study of the health status of individual woylies that have been trapped multiple times, and for which morphometric, haematological and disease data are available, would help assess the risks posed by specific factors to the health of individuals and populations, which

may be difficult to appreciate with a "one off" assessment. Such analyses may identify important cohorts of individuals for which the use of the banked samples may be recommended (to generate a complete disease dataset). Specific details for refinement and improvement of the health analysis carried out in this study are presented in Chapter 3. Additionally, analysis of sympatric species' demography and body condition data could also be valuable in generating further hypotheses.

Genetic parameters should also be included when carrying out the fitness and health investigations mentioned above (Acevedo-Whitehouse & Cunningham 2006). The interplay between the individual (host) genetic profile and disease susceptibility and fitness (including fecundity and survival) is a field that is receiving increasing attention (e.g. Amos et al. 2001; Hansson & Westerberg 2002; Acevedo-Whitehouse et al. 2003; Luikart et al. 2008; Banks et al. 2010). A typical example in humans is the altered genotype frequencies at the gene for sickle cell anaemia (HBB) in areas where malaria is present as a consequence of the increased survival of heterozygous individuals (Falconer & Mackay 1996). An example in wildlife is the selection pressure exerted by hookworm (*Uncinaria* spp.) infections in Californian (*Zalophus californianus*) and New Zealand (*Phocarctos hookeri*) sea lions (Acevedo-Whitehouse et al. 2006; Acevedo-Whitehouse et al. 2009). Similarly, in a woylie population the identification of a gene under selection pressure by a pathogen would justify a comprehensive investigation of the significance of that pathogen (Acevedo-Whitehouse & Cunningham 2006). If an aetiological agent is responsible for selection, it would be highly likely that it is also a relevant component of the regulation of the demography of the population (i.e. if the effect of the infection is irrelevant, it would be expected not to be responsible for genetic selection). This kind of information would also be important for active genetic management of captive breeding programs, although the complete removal of disadvantageous alleles is not recommended since, as shown by the example of genetically mediated resistance to malaria parasite infection

in humans (Falconer & Mackay 1996), a disadvantageous allele in one environment may become advantageous in another.

Lastly, unravelling an association of genome-wide heterozygosity and fitness (if present) would provide a quantified estimate of the potential of inbreeding depression in small populations (e.g. Coltman et al. 1998; Spielman et al. 2004; Charpentier et al. 2008; Luikart et al. 2008; Banks et al. 2010) and improve the PVA (by providing more realistic parameters).

A quantification of the influence of environmental factors, such as rainfall and diet, on haematological parameters should also be conducted in order to facilitate the understanding of the role of these variables on woylie fitness and to identify confounding factors. Exposure to biological vectors (e.g. arthropods) could also represent an important environmental component and its quantification may be pertinent. Serological screenings for common arboviruses known to occur in the area in other sympatric native species may provide further insight on this matter. In regard to this, collaboration with Dr Cheryl Johansen, who leads the Arbovirus surveillance project at the University of Western Australia (Department of Microbiology), has been established and arrangements have been made for an initial screening of Alphaviruses and Flaviviruses in woylie serum samples.

The disease risk assessment carried out in this study (Chapter 2) should not be considered final, but rather a dynamic process. When the risk assessment was carried out, several research projects were already ongoing and the aim was to establish what additional diseases should be considered as potentially responsible for the woylie decline. The disease risk assessment should be revisited in light of the most recent results of the other research components of the WCRP, to help define future priorities and allocation of resources.

10.5.2 Conservation genetics

Regular genetic monitoring is recommended for the benefit of species conservation (see Section 10.1). Relating this information to the baseline data generated by this study and the associated ecological and demographic data available would also provide an optimal opportunity to improve the understanding of genetic consequences of rapid population declines. This monitoring may help to quantify the genetic loss associated with the decline and evaluate the accuracy of PVA predictions. It might be possible to assess whether the spatial dynamics detected in this study will change when modifications of population densities occur. Also, it may help in the assessment of the success of management actions (e.g. supplementations) and detect if and when inbreeding depression becomes manifest in populations at lower genetic diversity (e.g. Tutanning).

Evaluation of historical samples, such as museum and/or fossil specimens, would allow measurement of the genetic material lost as a result of the restriction in the woylie distribution consequent to settlement by Europeans and quantifying the extent of the connections between woylie populations on a broader geographical scale. The evident historical connections between populations as far as 150 km apart suggest that the south west of Western Australia was a dynamic system. It is expected that the "functional" effective population size (the sum of the single effective population size of each unit) of this system was much larger than the one present nowadays (even at peak density). Consequently, it would be expected that the genetic diversity of the woylie was much larger. In collaboration with Dr Mike Bunce (Ancient DNA, School of Biological Sciences and Biotechnology, Murdoch University), preliminary tests were conducted on five museum and fossil specimens spanning from 40 to 12,000 years old. At least six microsatellite primer sets amplified detectable and

readable products on the fragment analyser and it was concluded that DNA preservation was of a sufficient quality to enable such an investigation to be undertaken.

10.6 Conclusions

In conclusion, this study provides evidence that diseases can not be dismissed as a possible cause or co-factor of the decline in woylies, despite the fact that no specific agent, or clinical process, was identified. It is most likely that no single cause is responsible for the decline and that the interplay of several factors (e.g. disease, predation) have resulted in being detrimental for woylie populations.

Several health parameters were described including genetic and haematological characteristics of woylies. The molecular genetic analysis allowed the identification of populations at risk of substantial loss of genetic diversity and possibly inbreeding depression. Additionally, historical and modern population dynamics, as well as genetic consequences of dispersal, were disclosed and the spatial organisation of woylie populations described. Lastly, the impact of different factors on genetic diversity and population demography was investigated, the minimum mortality rates that are necessary for the decline to occur were quantified and the minimum viable population size was estimated. The results of this study provide a reference point for future investigations into woylie health and genetics and can be used to inform management decisions as well as suggestions for future research.

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Appendix 1

Summary of groups of analyses and total number of tests used to protect against type I error

Variable of interest	Analysis	Number of tests (m)
Biometric index	Comparison between genders within age class	1
	Comparison by gender between locations within age class	2
	Comparison between reproductive status	1
	Comparison by gender between populations within age class	2
	Comparison by population and gender between seasons (adults only)	4
Haematological parameters	Comparison between gender within Karakamia	1
	Comparison between gender within Upper Warren	1
	Comparison by gender between locations	2
	Comparison by gender between populations	2
	Comparison by population and gender between seasons (Upper Warren)	4
	Comparison between seasons (Karakamia)	1
	Comparison by sex between pre and during decline	2
	Correlation with population abundance and rate of decline	20
	Comparison by population and gender between health problem (presence/absence)	4
Health problem prevalence	Comparison of health problem prevalence between locations	1
	Comparison of health problem prevalence between populations	1
	Comparison of health problem prevalence by gender between populations	3
	Comparison of health problem prevalence between populations within Upper Warren	1
	Comparison of health problem prevalence between forest blocks	3
	Comparison of health problem prevalence by forest blocks over time	3
	Comparison of health problem categories over time	1
	Correlation with population abundance and rate of decline	5

Appendix 2

Details of the multiplex approach for the 12 microsatellite loci amplified in woylie (*Bettongia penicillata*). Lab/Unlab refers to the proportion of labelled to unlabelled primers. *T*m: Annealing temperature. TD: touchdown.

Source species locus	Label	Lab/ Unlab	*T*m	Multiplex	Post-PCR mix group
Bettongia tropica					
Bt64	Fam	1/2	TD	M3	B
Bt76	Fam	1/2	61	M4	A
Bt80	Vic	1/2	61	M4	A
Petrogale assimilis					
Pa593	Vic	1/2	57	Singleplex	B
Petrogale xanthopus					
Y105	Vic	1/3	61	M15	A
Y112	Pet	1/3	61	M15	A
Y151	Ned	1/2	57	Singleplex	B
Y170	Vic	1/3	TD	M10	A
Y175	Fam	1/2	TD	M3	B
Potorous longipes					
Pl2	Pet	1/2	61	M4	A
Pl26	Ned	1/3	TD	M10	A
Macropus eugenii					
T17-2	Fam	1/2	TD	M3	B

Appendix 3

Details of woylie individuals indentified as migrants between populations under the admixture model with correlated allele frequencies without geographic information.

ID	Sex	(%Miss)[a]	Into	From	Generation[b]	P(*i*)[c]	P(*f*)[d]
07-354	M	0	Boyicup	Dryandra	F2	0.508	0.281
07-370	M	0	Corbal	Dryandra	F2	0.579	0.412
07-580	M	8	Keninup	Dryandra	F2	0.672	0.314
07-685	M	8	Yendicup	Dryandra	F2	0.685	0.267
08-016	F	0	Yendicup	Dryandra	F2	0.578	0.351
07-341	F	0	Balban	Kingston	F1	0.296	0.664
07-351	M	0	Balban	Kingston	F2	0.624	0.357
07-389	M	0	Chariup	Kingston	F0	0.007	0.988
07-585	M	0	Moopinup	Kingston	F0	0.005	0.862
07-587	F	0	Moopinup	Kingston	F1	0.121	0.582
07-366	M	0	Corbal	Perup	F2	0.599	0.393
07-381	F	0	Corbal	Perup	F2	0.738	0.221
07-680	M	8	Warrup	Perup	F2	0.598	0.332

[a] percentage of genetic data missing.

[b] Generation was established based on *P(f)* as follows:F0, *P(f)*>0.85; F1, *P(f)>0.45; F2, P(f)>0.2*

[c] Posterior mean estimate of the proportion of the genome inherited from the putative population (i.e. where sampled).

[d] Posterior mean estimate of the proportion of the genome inherited from the source population (i.e. where sampled)

Appendix 4

Summary of genotyped animals from each forest block in Upper Warren

Population	Block	Sample size
Perup	Balban	37
	Boyicup	6
	Chariup	9
	Dordagup	1
	Dwalgan	1
	Keninup	31
	Moopinup	6
	Poorginup	1
	Yendicup	10
Kingston	Corbal	26
	Warrup	31
	Winnejup	12