ACTIVE DISEASE SURVEILLANCE IN KANGAROOS UTILISING THE COMMERCIAL HARVESTING INDUSTRY

Presented by

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Declaration

This thesis constitutes work carried out by the candidate unless otherwise stated and does not incorporate without acknowledgement any material previously submitted for a degree at any tertiary institution. The thesis is less than 100,000 words in length, exclusive of tables, figures, bibliography and appendices and complies with the stipulations set out for the degree if Doctor of Philosophy by Murdoch University.

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Aims

1. To develop a framework for active disease surveillance in kangaroos using the commercial harvesting industry.

2. To determine the prevalence of naturally acquired *Salmonella* infection in wild kangaroos in Western Australia.

3. To further define the role of the kangaroo as reservoir host of *Coxiella burnetii* in Western Australia.

4. To further define the role of the western grey kangaroo as reservoir host of Ross River virus and determine whether ongoing surveillance will improve the capacity to predict periods of increased viral activity.

Thesis Abstract

The aim of this study was to develop a framework for disease surveillance in one of the Australia's most abundant macropods using the kangaroo harvesting industry. The impetus for this work arose because wildlife species are considered to play a significant role in the introduction, maintenance and spread of a majority of the world's emerging infectious diseases yet active disease surveillance is rarely undertaken in these free-ranging populations. The framework developed was trialled by collecting samples and testing them for a number of significant emerging infectious diseases, including *Salmonella, Coxiella burnetii* and Ross River virus (RRV).

Kangaroos have long been suspected of carrying high levels of *Salmonella,* yet no definitive study has been undertaken to determine the true prevalence of infection in their natural habitat. Faecal samples were collected from 645 western grey kangaroos (*Macropus fuliginosus*) from ten different geographical locations throughout Western Australia over a period of 18 months and cultured for *Salmonella* spp. The estimated prevalence in the animals surveyed was approximately 3.6%. Faecal shedding was greatest following increased periods of rainfall in the April to June quarter. The relatively low prevalence of faecal shedding suggests that kangaroos in their natural habitat support the organism but are unlikely to pose any greater risk of zoonotic infection than other domestic livestock species. Whilst kangaroos have not yet been associated with food-borne outbreaks of disease, serotypes known to cause salmonellosis were isolated in this study, such as *Salmonella enterica* serovar Muenchen, Kiambu and Saintpaul.

Few studies have investigated the role of macropods in the maintenance and transmission of *C. burnetii*. Paired faecal and serum samples were collected from approximately 1000 western grey kangaroos from across twelve locations throughout Western Australia. An indirect ELISA was used to detect *C. burnetii* antibodies in serum, whilst quantitative PCR was used to detect *C. burnetii* DNA in faecal material. The estimated seroprevalence across all sample collection sites was 24.1%, whilst *C. burnetii* DNA was detected in the faeces of 4.1% of animals surveyed. Seroprevalence was significantly higher following increased periods of rainfall in the 60 days prior to sample collection ($p<0.05$), with seroprevalence lowest in the October to December quarter. These results suggest that kangaroos are likely reservoirs of the organism in Western Australia, posing a zoonotic threat to industry workers and animal handlers.

Ross River virus is Australia's most common mosquito-borne disease and the western grey kangaroo is suspected of being a significant vertebrate host in the southwest of Western Australia. A total of 2605 serum samples, collected from across fourteen locations throughout the state, were tested for RRV neutralising antibodies. The seroprevalence varied significantly between geographical regions but was estimated to be 44.0% across all sample collection locations. Despite difficulties associated with age-based selection bias introduced through the kangaroo harvesting industry, surveillance within western grey kangaroo populations appears to provide a means of assessing the background risk of RRV for any given location and may assist in improving the capacity to predict future RRV activity.

Abbreviations

<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
±	plus or minus
%	percent
°C	degrees Celcius
µl	microlitre
AB-CRC	Australian Biosecurity Cooperative Research Centre
ABS	Australia Bureau of Statistics
ADE	antibody dependent enhancement
Apr	April
ASRL	Arbovirus Surveillance and Research Laboratory
AWHN	Australian Wildlife Health Network
BOM	Bureau of Meteorology
CALM	Conservation and Land Management
CFT	complement-fixation test
CI	confidence interval
cm^2	centimetres squared
Dec	December
DEC	Department of Environment and Conservation
dd	double distilled
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetra acetic acid, tri-potassium salt
EID	emerging infectious disease
ELISA	enzyme-linked immunosorbent assay
et al.	and others
EVS	encephalitis vector survey
FBS	foetal bovine serum
FMD	Foot and mouth disease
g	grams
g	unit of gravitation field

G	gauge
GIS	geographical information system
H_2O	water
HI	haemagglutination-inhibition
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
Jan	January
Jun	June
Jul	July
kg	kilogram
KMAC	Kangaroo Management Advisory Committee
L	litre
LCV	large cell variant
m	metre
M	molar concentration
Mar	March
MBDC	Mosquito-Borne Disease Control Branch (Department of Health)
min	minute
MIR	minimum infection rate
ml	millilitre
mm	millimetre
n	number of animals
NC	not calculated
ND	not detected
NDVI	normalised difference vegetation index
NEPSS	National Enteric Pathogens Surveillance Scheme
NSW	New South Wales
NT	neutralisation test
NTC	no template controls
Oct	October
OD	optical density
OR	odds ratio

PP	percent positive
QLD	Queensland
qPCR	quantitative polymerase chain reaction
RainCat30	accumulated rainfall in the previous 30 days
RainCat60	accumulated rainfall in the previous 60 days
RRV	Ross River virus
SA	South Australia
Sept	September
SCV	small cell variant
SDC	small dense cell
$TCID_{50}$	50% tissue culture infectious dose
TEN-T	TE and NaCl with 0.05% (v/v) Tween 20
VIC	Victoria
v/v	volume in volume
WA	Western Australia
WGK	wester grey kangaroo (*Macropus fuliginosus*)
w/v	weight in volume

1. LITERATURE REVIEW

1.1. Introduction

This literature review covers a broad range of topics including disease surveillance in wildlife, the kangaroo harvesting industry and significant infectious organisms of macropods; *Salmonella, Coxiella burnetii* and Ross River virus. Various ecological aspects relevant to the epidemiology of these aforementioned organisms will also be discussed.

1.2. Global Emergence of Infectious Disease

Emerging infectious diseases (EIDs) are defined as 'infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range' (Morse 1995). Over the past two decades, emerging infectious disease has been cited as the number one cause of death in humans around the world (National Institute of Allergy and Infectious Disease 2007). Approximately 75% of the diseases listed as 'emerging' pose a zoonotic threat and many have the potential to be used as weapons in bio-warfare (Chomel, Belotto et al. 2007).

Whilst EIDs differ in terms of their causative agent, the species affected and manifesting clinical signs, a common element that exists between them is the significant role wildlife species play as reservoir hosts. Prominent examples include Rabies, Hendra virus, Avian Influenza, Australian bat lyssavirus, SARS and Nipah virus (Williams, Yuill et al. 2002). Yet despite such knowledge, very few structured surveillance programs exist for wild animal populations. Adequate time and financial resources are generally only spent on disease surveillance in wildlife when there is a

risk of a significant impact on the economy, livestock or human health (Daszak, Cunningham et al. 2000). With increased international travel, animal movement, bioterrorism and greater contact between humans, domestic stock and wildlife, there has been a call in Australia to establish monitoring programs that assist in the early recognition and control of exotic, zoonotic and locally devastating disease (Australian Wildlife Health Network 2006; Department of Agriculture Fisheries and Forestry 2006).

1.2.1. Factors Influencing the Emergence of Infectious Diseases

Improved diagnostic tools and increased vigilance do not completely explain the increase in the number of EIDs over the past two decades (Daszak, Cunningham et al. 2000; Chomel, Belotto et al. 2007). A set of causal factors, many anthropogenic, can be identified for almost all situations where an infectious disease is considered to be 'emerging' (Morse 1995). These include:

- Ecological change
- Agricultural practices
- Increased travel/transportation of both humans, animals and vectors
- Microbial adaptation
- Human demographics and behaviour

Increased human-wildlife contact is a common outcome of many of the factors listed above. As urban sprawl continues to encroach upon shrinking wildlife habitats, human contact with wild animal populations continues to increase (Chomel, Belotto et al. 2007). Deforestation, mining and urban development practices all add to this pressure as reduced living space leads to an increase in population density in wildlife (Daszak, Cunningham et al. 2000). In countries such as China, multi-species farming and

mixing of domestic stock with wildlife has become common practice. This provides a "natural laboratory" for new viral recombinants, particularly in the case of diseases such as influenza (Scholtissek and Naylor 1988). More recently, people have taken a keen interest in keeping wildlife as pets, particularly in the United States of America (Marano, Arguin et al. 2006). This close interaction not only increases the risk of disease transmission but also increases the risk of exotic disease introduction. Ecotourism is also becoming extremely popular (Chomel, Belotto et al. 2007). Wildlife parks, zoos, walk-through animal houses and mobile petting farms all encourage interaction with animals. Commercial and recreational hunting of wildlife species and consumpion of bushmeat provides another means of increasing the exposure of humans potential disease reservoirs (Chomel, Belotto et al. 2007).

1.2.2. Disease Surveillance in Wildlife

Surveillance of wildlife presents many unique problems that epidemiologists do not generally encounter when undertaking surveillance of domestic livestock. It is very difficult to predict both movement and interaction of free-ranging animals. Logistical challenges also exist in humanely trapping, sampling and releasing a large enough sample population to provide statistically meaningful data (Pfeiffer and Hugh-Jones 2002). A growing awareness of the role that wildlife species play in infectious disease transmission has led to new methods of surveillance being employed to overcome these challenges. Hypothesis-driven surveillance is one such example, aimed at overcoming the financial and logistical constraints of surveillance over large temporal and spatial scales so often associated with wildlife populations (Hoye, Munster et al. 2010). This concept has been used in the surveillance of wild birds for avian influenza virus where standardised, local surveys are undertaken and then strategically compiled

over broader geographic areas. A key element of hypothesis-driven surveillance is the use of investigator-defined surveillance designs that consider a compromise between sampling based on probability and and the constraints of sample collection, transport and analysis (Hoye, Munster et al. 2010).

The use of Geographical Information Systems (GIS) is also beneficial in wildlife surveillance, incorporating spatial relationships into epidemiological investigations of wildlife disease (Pfeiffer and Hugh-Jones 2002). Complex datasets describing different aspects of the behavioural tendencies and environment of wild animals can be integrated (Pfeiffer and Hugh-Jones 2002). Analysis using GIS can be used to visualise, explore or model the various stages involved in surveillance of disease, including case prediction, risk assessment and control programme implementation (Pfeiffer and Hugh-Jones 2002). One such study used GIS to map the prevalence of IgG antibodies against *Coxiella burnetii* in Cyprus and in doing so, identified regions that were considered high risk for Q fever transmission (Psaroulaki, Hadjichristodoulou et al. 2006).

1.2.2.1. Disease Surveillance in Wildlife in Australia

Australia is one of the most geographically isolated continents in the world hence it is more difficult for an exotic disease to be introduced through shared water bodies, animal migration or wind from neighbouring countries (Gee 1982). Despite this physical barrier, diseases may still be introduced through the movement of infected people, vectors, fomites, animals and animal by-products. Indeed, highly contagious H3 equine influenza was introduced into the domestic horse population in Australia in 2007 as a result of the failure to adequately quarantine an imported horse (Hammond

2007). Furthermore, newly recognised pathogens such as Hendra virus, viral chorioretinitis and Australian bat lyssavirus have recently been identified in Australia (Daszak, Cunningham et al. 2000).

The Australian Wildlife Health Network (AWHN) was established in 2002 to improve disease surveillance in wild animal populations (Australian Wildlife Health Network 2006). The group currently coordinates wildlife health surveillance across the country and aims to provide 'a nationally integrated wildlife health system for Australia' (Australian Wildlife Health Network 2006). Roles include managing emergency animal disease preparedness programs, compiling the Wildlife Health Information System and conducting biosecurity training (Australian Wildlife Health Network 2006). A weekly on-line bulletin is emailed to all members of the network providing an update on wildlife diseases of national and international biosecurity importance.

1.2.2.2. Disease Surveillance in Wildlife in Western Australia

In Western Australia the Department of Agriculture and Food is responsible for disease surveillance in all animals, including wildlife. National and state-based activities to prevent exotic disease introduction are coordinated through the Wildlife Exotic Disease Preparedness Program, which has been very effective in 'generating awareness of the potential role of wildlife and feral animals in the spread of exotic diseases' (Wells, Russell et al. 1993; Ryan, Do et al. 2000; Department of Agriculture 2007). Passive surveillance is the primary form of disease monitoring undertaken in wildlife, which relies on the reporting of diseased animals to the Department by individual members of the public or relevant government agencies. Few diseases in native West Australian animal populations are classified as 'notifiable', so there is no

formal obligation for anyone to report suspicious clinical signs (Department of Agriculture Fisheries and Forestry 2006). The efficiency of this system is likely to be low given that there is no systematic process of monitoring or observing wildlife populations and little motivation for individuals to report wildlife cases to the relevant authorities.

1.3. Natural History and Ecology of the Kangaroo

The kangaroo belongs to the Superfamily Macropodoidea, of which the largest family are the Macropods, consisting of kangaroos and wallabies (Dawson 2002). At the time of European settlement there were 50 species of macropods in Australia, 23 of which were found within Western Australia (Department of Conservation and Land Management 2002). According to the *Wildlife Conservation Notice 2002*, 14 species are still considered to be in abundance in WA. The remainder have decreased in number and now vary in conservation status from threatened to extinct. Nationwide, there are four groups of kangaroos; the greys, the reds, the antilopine kangaroos and the wallaroo-euro group (Dawson 2002). In Western Australia, the red kangaroo (*Macropus rufus)* is the most abundant species, followed by the western grey kangaroo (WGK) (*Macropus fuliginosus)*. In the years following European settlement, western grey and red kangaroo populations flourished due to increased access to pastures associated with farming and the introduction of fox-baiting programs, which removed predators (Department of Conservation and Land Management 2002).

1.3.1. Distribution of the Red and Western Grey Kangaroo in Western Australia

The WGK is predominantly found in the southern regions of Australia with the highest densities recorded in the southwest (Department of Environment and Conservation 2007) (*Figure 1.1*). The red kangaroo is present in all regions except the northern Kimberley and the southwest of the state (Department of Environment and Conservation 2007; 2008) and is most commonly found in central Western Australia where rainfall is less than 500mm per year (Dawson 2002) (*Figure 1.2*). Both species occur together in the central to southeast region of the state.

1.3.2. Home Range and Habits of Kangaroos in Western Australia

Dawson (2002) defined a kangaroo's home range as 'an area of land covered on a regular basis in order to feed, mate and care for young'. Kangaroos are relatively sedentary in nature, particularly as they mature. In the *Central* and *Northern Zones,* where the country is more open, home ranges are a little more variable or 'drifting'. Kangaroos may be forced to move further in these regions as a consequence of unfavourable environmental conditions and to search for both food and water (Dawson 2002).

Western grey kangaroos have been shown to utilise a relatively small home range (Priddel, Shepherd et al. 1988). These animals tend to utilise a core area of land during the day, predominantly for rest, and a separate core area at night, for grazing (Dawson 2002). Despite drought-induced feed shortages, a study in western New South Wales demonstrated that more than 90% of monitored WGKs remained within a distance of six kilometres over a period of 18 months (Priddel, Wellard et al. 1988).

Figure 1.1 Distribution of the western grey kangaroo (*M. fuliginosus)* in Western Australia

(Department of Environment and Conservation 2008)

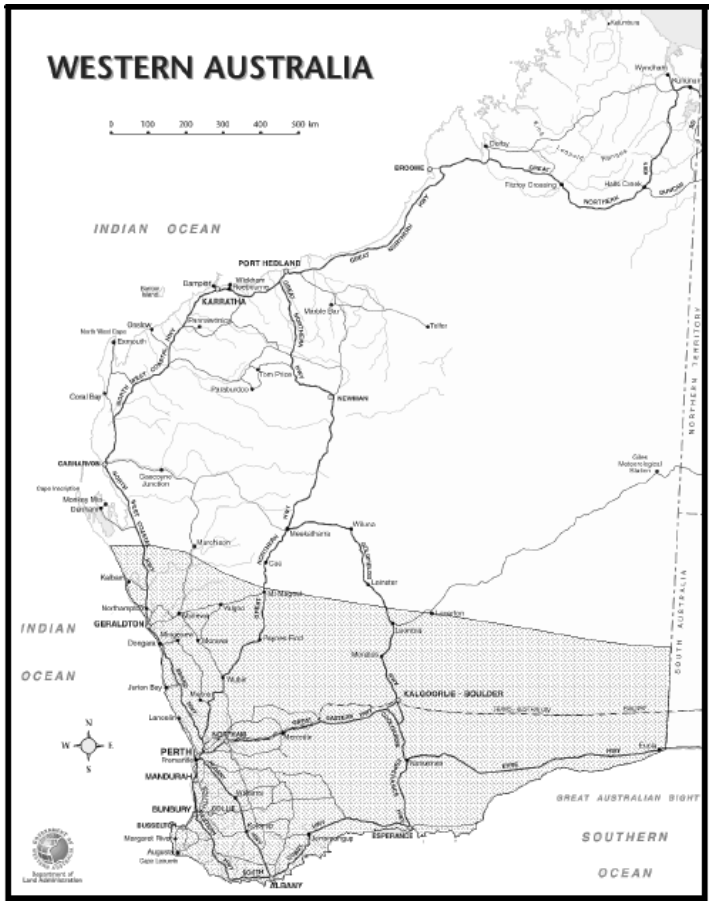
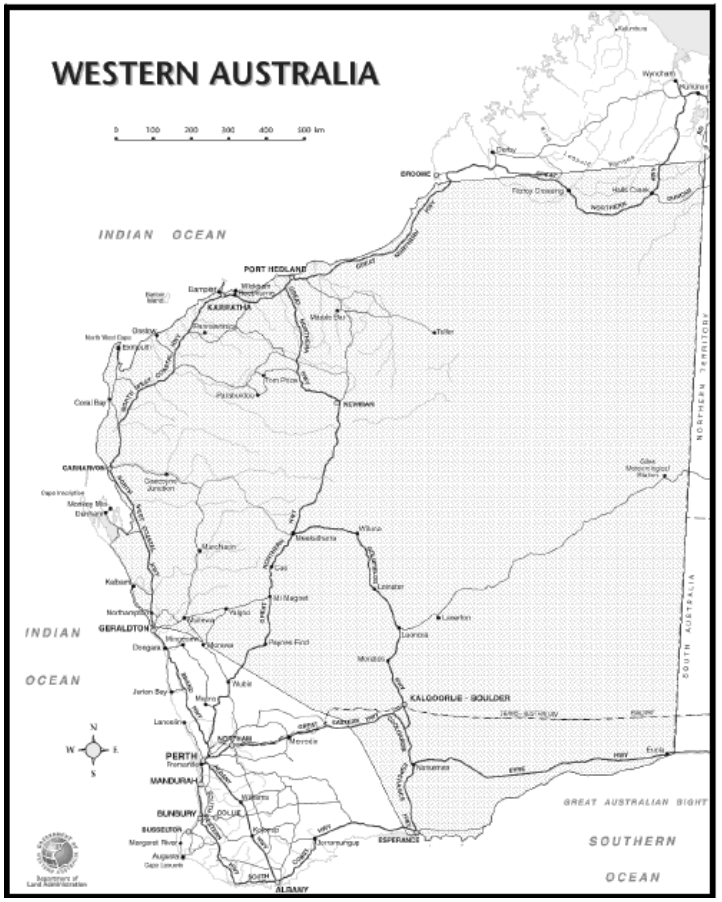


Figure 1.2 Distribution of the red kangaroo (*M. rufus*) in Western Australia

(Department of Environment and Conservation 2008)



A second study in southern Western Australia, reported that less than 2.5% of monitored western grey kangaroos travelled outside of a study region, with a radius of 1.6 kilometres (Arnold, Grassia et al. 1991). In the latter study, 9 out of 10 of the animals that left the study were male (Arnold, Grassia et al. 1991). Given these animals graze between 5.9 and 9.8 hours per day, it is not surprising that they do not travel long distances (Priddel 1986). A significant proportion of grazing activity (86%) occurs in the hours prior to sunrise and following sunset (Priddel 1986). Western grey kangaroos spend a relatively consistent amount of time grazing in autumn, winter and spring. Grazing time decreases in summer due to the warmer weather, when kangaroos seek shade to rest (Priddel 1986).

The home range of the red kangaroo is larger than the home range reported for the WGK (Priddel, Wellard et al. 1988) but is considered to be relatively fixed (Dawson 2002). Movement of red kangaroos is generally restricted to an area less than $10km^2$ (Priddel, Shepherd et al. 1988; Department of Environment and Conservation 2008). Croft (1991) reported that the daily home range of the red kangaroo was approximately 1.5 km^2, increasing two- to three-fold when considering movements over the period of a week(Croft 1991). Oliver (1986) suggested that the distinct coat colour in red kangaroos in different, adjacent districts was evidence of the sedentary nature of this species. Movement over significant distances would have led to inter-breeding and more a homogenous coat colour (Oliver 1986). Norbury et al. (1994) recorded a more variable home range for the red kangaroo in arid Western Australia, averaging $18.4km^2 \pm 5.0km^2$ for adult females and $36.1km^2 \pm 17.2km^2$ for adult males. These are by far the largest home ranges noted by any study and are possibly due to changes in the home range of the studied population for environmental or

social reasons. In times of feed shortage, this may be a temporary move or one that is undertaken on a daily basis (Dawson 2002).

1.3.3. Sex Structure

Kangaroos exhibit a degree of sexual segregation due to sex-specific differences associated with body size and reproductive strategies (MacFarlane and Coulson 2007). Whilst females exist in greater numbers within mobs, males remain the dominant sex. Johnson and Jarman (1983) reviewed the results of fifteen studies between 1964 and 1982, and concluded that there was no statistical difference in the birth ratio of male to female pouch young. These findings were later supported by the results of studies undertaken by Arnold et al. (1991) and Norbury et al. (1988).

A similar balance between the number of males and females exists in the subadult age bracket (Newsome 1977; Norbury, Coulson et al. 1988). The sex ratio in adult kangaroos is consistently female biased (Newsome 1977; Johnson and Bayliss 1981; Norbury, Coulson et al. 1988). Norbury et al. (1988) found that the ratio of male to female, adult WGKs in Hattah-Kulkyne National Park, Victoria, over a 3 year period, was 1:3 (Norbury, Coulson et al. 1988). A number of subsequent studies supported these observations (Newsome 1977; Johnson and Bayliss 1981). In contrast, Arnold et al. (1991) noted that 46.8% of the adult WGK population at Baker's Hill in WA was male, suggesting that the sex ratio in kangaroos is not consistent across all areas of Australia. This discrepancy may be due to differences in sampling methodologies, with Arnold et al. (1991) relying on visual observation of live animals, not lethal shooting. Caution must be exercised in accepting the results of this study because there is a risk of wrongly sexing a kangaroo from a distance in

observation studies. Furthermore, behavioural differences between males and females may influence whether they are easily observed or not.

The shift from an evenly balanced number of male and female kangaroos in the subadult age bracket to a female dominated adult population can be explained by male biased mortality in adult animals. Norbury et al. (1988) observed that the female biased sex ratio developed after 3 – 5 years of age and continued to increase with age until only 19% of 9-11 years olds were male (Norbury, Coulson et al. 1988). Whilst this may be partially attributed to the selective harvesting of male kangaroos, the increased nutritional requirement of male kangaroos may also result in a greater number of male deaths during times of drought (Newsome 1977). On average, male kangaroos graze for longer than females each day (Priddel 1986). When rainfall is low and feed shortages ensue, the older male kangaroos are one of the first groups to be affected (Newsome 1977; Norbury, Coulson et al. 1988; Arnold, Grassia et al. 1991). During the 1982 drought in New South Wales, kangaroo populations dropped by 43% (Caughley, Bayliss et al. 1984). In Kinchega National Park, it was estimated that 30% of red and 67% of grey kangaroos died due to harsh environmental conditions (Robertson 1986). The majority of animals affected were either subadults of either sex or older males. Grey kangaroos began dying before red kangaroos, but as the drought progressed, red kangaroos were increasingly affected (Robertson 1986).

1.3.4. Age Structure in Kangaroos

Determining the age structure in a population of kangaroos requires consideration of both tooth eruption, measurements of body size and proportions (Dawson 2002). The age structure of kangaroo mobs generally tends toward a pyramidal structure, where

frequency of older animals in the adult age bracket declines with age (Norbury, Coulson et al. 1988). Norbury et al. (1988) reported a ratio of subadult (1-3 years old) to adult kangaroos of 1:2.5 or approximately 28.6% of the total population. Arnold et al. (1991) similarly found that 26.3% of the kangaroo population at Baker's Hill in WA, between 1977 and 1985, were subadult or juvenile, although this varied between 10.7% and 36.2% of the population depending on the year (Arnold, Grassia et al. 1991).

Drought has a profound effect on the age structure of a population through its influence on both the birth and death rate in kangaroos. Abundance of rainfall, followed by increased food supply, is an important factor in the production of young (Newsome 1977; Bayliss 1985; Norbury, Coulson et al. 1988; Arnold, Grassia et al. 1991). This is particularly so for the WGK, whose strict breeding season and rate of reproduction are dictated by feed abundance (Bayliss 1985; Dawson 2002). At Hattah-Kulkyne National Park, Victoria, the number of mature females that were breeding dropped to 46% in the three years following a severe drought. With the return of rainfall, this increased to 100% two years later (Norbury, Coulson et al. 1988). Drought also has a significant impact on the mortality rate in kangaroos. Arnold et al. (1991) estimated that on average only 27% of young emerging from the pouch survived their first year. During times of drought, mortality is even higher, particularly in the subadult (Robertson 1986), pouch young (Newsome 1965) and older male age groups (Robertson 1986). Consequently, variation in rainfall and feed supply can have a significant impact on the long term population structure of the kangaroo (Newsome 1977). Peaks and troughs in both the birth and death rates can

potentially result in an inverted age structure as shown by Newsome's (1977) study of the red kangaroo.

1.3.5. Reproduction in Kangaroos

Macropod reproduction differs from other mammal reproduction in that the gestation period is comparatively short and many species exhibit embryonic diapause (Dawson 2002). The red kangaroo is a continuous, opportunistic breeder, influenced by both environmental conditions and feed availability (Bayliss 1985). Birth occurs just prior to the start of the next oestrous, with the young making its way to the pouch to suckle. Very soon after, the oestrous cycle begins again and post-partum mating occurs. The new embryo then enters diapause, which results in the suspension of growth at the blastocyst stage. The embryo will not resume development if the newborn young reaches the pouch and lactation begins (Dawson 2002). Red kangaroos have a mean gestation period of 33.2. Young exit the pouch for the first time at 185 days and are weaned at 540 days following birth (Dawson 2002).

The WGK tends to be a seasonal breeder and is unique to the Macropod family in that it does not experience embryonic diapause (Poole and Catling 1974; Bayliss 1985; Dawson 2002). Successful mating will only occur after weaning of the pouch young is complete (Dawson 2002). Most young are born between September and March, ensuring feed in abundant for lactating does during the autumn and winter months and emerging pouch young during late spring (Norbury, Coulson et al. 1988; Arnold, Grassia et al. 1991; Dawson 2002). Western grey kangaroos have a mean gestation period of 30.6 days. Young exit the pouch for the first time at 298 days and are weaned at 360 days following birth (Dawson 2002).

Sexual maturity in female and male red kangaroos occurs at around 15 – 20 months and 24 months of age, respectively (Dawson 2002). Female WGKs do not reach sexual maturity until at least 14 – 16 months of age and often do not reproduce for the first time until some time later (Norbury, Coulson et al. 1988; Dawson 2002). By weight, the female WGK reaches sexual maturity at approximately 16kg. Sexual activity peaks from 24kg onwards, which is estimated to be approximately three years of age (Arnold, Grassia et al. 1991). Male WGKs are approximately 30 months old when they reach sexual maturity (Poole and Catling 1974; Norbury, Coulson et al. 1988). Although the majority of male WGKs are considered adult once they reach a body weight of 45 – 50 kg, they do not contribute significantly to the breeding cycle until they are 55 – 60 kg (Dawson 2002). Sexual maturity is delayed by approximately 6 months during drought (Newsome 1965; Dawson 2002).

1.4. The Kangaroo Harvesting Industry

The kangaroo harvesting industry was established in Australia almost 40 years ago to reduce the damage to farm fences, crops and pastures caused by increasing kangaroo populations (Pople and Grigg 1999; Kangaroo Industry Association of Australia 2006). In more recent years kangaroo meat and skins have become an important economic resource both locally and overseas (Kelly 2002).

Six species of macropod are currently harvested in Australia (*Table 1.1*). The eastern grey (*Macropus giganteus*), western grey (*Macropus fuliginosus*) and red kangaroo (*Macropus rufus)* constitute 90-95% of the total commercial harvest nation wide (Pople and Grigg 1999; Department of Environment Water Heritage and the Arts

2007), with the latter two forming the majority of Western Australia's harvest. The euro (*Macropus robustus)* is harvested intermittently in WA when populations are considered adequate. Harvesting of the euro is not governed by the same regulations as the red and WGK (Department of the Environment Water Heritage and the Arts 2007) and will not be considered further for the purpose of this project.

Table 1.1 Macropod species currently under harvest management in Australia (Pople and Grigg 1999; Department of Environment Water Heritage and the Arts 2007).

Species	Scientific Name	State
Red kangaroo	(*M. rufus*)	QLD, NSW, SA, WA
Eastern grey kangaroo	(*M. giganteus*)	QLD, NSW
Western grey kangaroo	(*M. fuliginosus*)	NSW, SA, WA
Common wallaroo/euro	(*M. robustus*)	QLD, NSW, SA
Bennetts wallaby	(*M. rufogriseus*)	TAS (Flinders & King Island)
Tasmanian pademelon	(*Thylogale billardierii*)	TAS (Flinders Island)

1.4.1. Management of the Kangaroo Harvesting Industry in Western Australia

The Department of Environment and Conservation (DEC) manages all aspects of the kangaroo harvesting industry in Western Australia. Every five years, DEC must produce a written *Management Plan* aimed at sustaining populations of red kangaroos and WGKs, whilst managing them as a renewable resource (RSPCA 2002). The document must satisfy the requirements of the *Environment, Protection and Biodiversity Conservation Act 1999* and be approved by the Commonwealth Minister

for the Environment and Water Resources and Scientific Committee for Sustainable Use of Wildlife (Department of Conservation and Land Management 2002; Department of Environment and Conservation 2008; Department of the Environment Water Heritage and the Arts 2008). The aims and technical management of the kangaroo harvesting industry are explained in the *Management Plan for the Commercial Harvest of Kangaroos in Western Australia, 2008 – 2012* (Department of Environment and Conservation 2008).

The Kangaroo Management Advisory Council (KMAC) was established in 1971 to ensure industry members, as well as DEC, played a role in making decisions that concerned Western Australia's commercial harvesting industry (Department of Conservation and Land Management 2002). The KMAC consists of representatives from DEC, the Department of Agriculture, Pastoralists and Graziers Association of Western Australia, Professional Shooters Association, The Western Australian Farmers Federation, kangaroo shooters and processors (Department of Conservation and Land Management 2002).

1.4.1.1. Legislation Governing the Kangaroo Harvesting Industry

The commercial kangaroo harvesting industry in Western Australia is regulated by a number of Federal and State Legislative Acts, Regulations and Codes that ensure its successful management. Whilst kangaroos are protected by section 14 of the *Wildlife Conservation Act 1950* (Department of Conservation and Land Management 2002; Australasian Legal Information Institute 2003), they are also considered a "Category A7" species under the *Agriculture and Related Resources Protection Act*, *Section 3*. This refers to native animals 'for which a management programme should, in the

opinion of the Protection Board, be approved' (Australasian Legal Information Institute 2004). Consequently, harvesting of kangaroos may take place in many areas of Western Australia considered to be *Open Season* (Australasian Legal Information Institute 2004). If shooters obtain an appropriate licence, they are able to commercially harvest kangaroos in these areas. *Closed Season* is allocated to areas in WA where kangaroo populations are considered to be lower. Shooting is generally not permitted here unless an additional *Damage Licence* is obtained from DEC (Department of Conservation and Land Management 2002).

1.4.1.2. Licensing of Shooters, Chillers, Processors and Skin Dealers

It is mandatory to obtain an appropriate licence to harvest kangaroos, to process them and to sell their skins (Department of Environment and Conservation 2008). Professional shooters must obtain a *Wildlife Conservation Regulation 6 Licence* which allows licensees to 'take kangaroos by means of a firearm on a property that falls within the area described in the *Open Season Notice for Western Grey Kangaroos'* and 'to sell the carcasses or skins to a Kangaroo Processor' (Department of Conservation and Land Management 2002). To become an accredited professional shooter, individuals must complete the "Australian Game Meat Hygiene and Handling" course by distance education (Technical and Further Education Commission NSW 1999; Department of Conservation and Land Management 2002). They must also hold a firearms licence and pass a firearms competency test (Department of Conservation and Land Management 2002). In 2008, there were 368 professional shooters licensed by DEC (Department of Environment and Conservation 2008). The number of licensed shooters fluctuates each year, but this number

generally falls between 360 and 390 individuals (Department of Environment and Conservation 2008).

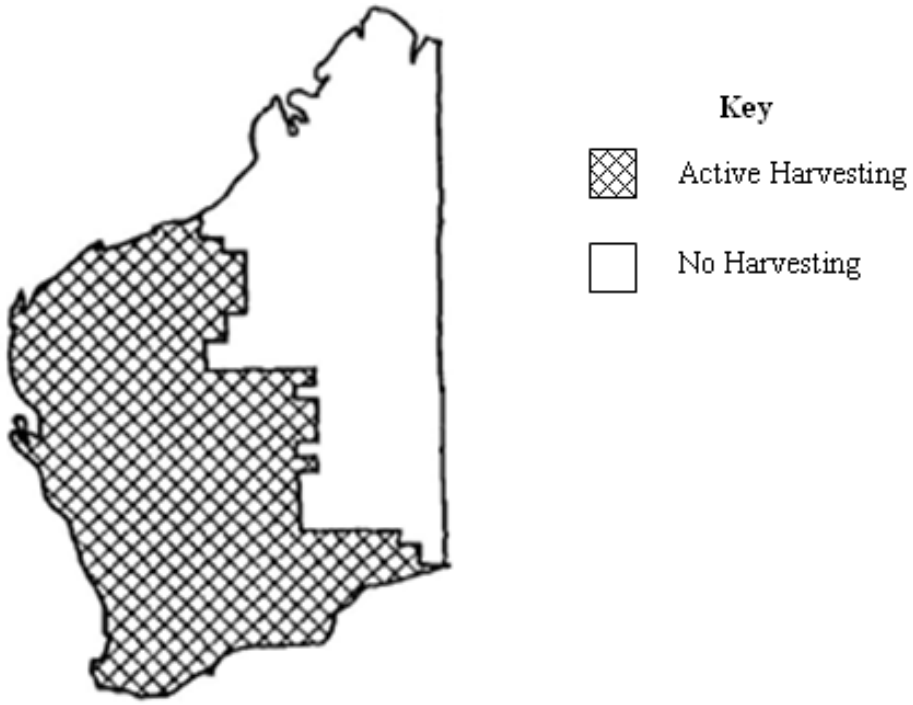
Professional shooters may only harvest kangaroos from properties where permission has been obtained from the primary landholder and where the property has been registered on the shooter's licence with DEC (Department of Conservation and Land Management 2002; RSPCA 2002). At the end of each night of harvesting, professional shooters must deliver all kangaroo carcasses to an approved chiller facility kept in the field or to the processor directly. At any point in time, shooting operations and chiller facilities may be inspected by DEC representatives. Kangaroo processors and skin dealers must also obtain an appropriate licence and abide by the relevant legislation in order to operate (Department of Environment and Conservation 2008).

The *Code of Practice for the Humane Shooting of Kangaroos* ensures that kangaroos are shot in a humane manner (Department of Environment and Heritage 1990). All professional shooters receive a copy of the *Code* when they become licensed and again when amendments are made to the document (Department of Conservation and Land Management 2002). The *Code* dictates that a single shot to the brain must be used to kill a kangaroo (Department of Environment and Heritage 1990). This ensures that death is instantaneous and suffering is minimal. If the initial shot is unsuccessful and the kangaroo is injured, it is acceptable to apply a blow to the back of the skull with a blunt instrument or to shoot the animal in the heart (Department of Environment and Heritage 1990).

1.4.2. The Geographical Boundaries of the Kangaroo Harvesting Industry in Western Australia

Kangaroos are harvested primarily in the pastoral grazing regions of Western Australia (Pople and Grigg 1999). Farmers permit shooters to enter onto their properties free of charge to reduce animal numbers. No commercial harvesting of kangaroos takes place in the Kimberley, the eastern Pilbara or a significant portion of the north-eastern Goldfields-Esperance region, indicated by the unshaded areas in *Figure 1.3*. This is because kangaroos numbers are either too small or the land is uninhabited and kangaroos shooting is not necessary.

Figure 1.3 Geographic boundaries of the kangaroo harvesting industry in Western Australia



1.4.3. Population Monitoring Methods

The Department of Environment and Conservation undertakes annual aerial surveys of Western Australia to monitor kangaroo populations. For this purpose, the state is geographically divided into four *Population Monitoring Zones; Central, South Eastern, South Western* and *Northern* (*Figure 1.4*). In 1981, DEC began extensive fixed wing, strip transect aerial surveys, covering approximately 900,000 km^2 on a triennial basis (Pople and Grigg 1999; Department of Conservation and Land Management 2002). In 1995, this system was replaced by a series of annual surveys designed to assist in the more accurate detection of population change. The *Central, South Eastern* and *Northern Zones* are surveyed extensively every three years, with a minimum of six, one-degree latitude by one degree longitude blocks monitored in the two years in between (Department of Conservation and Land Management 2002; Department of Environment and Conservation 2008). Aerial surveillance in this manner provides DEC with an annual update on population trends. The flight lines used to undertake population surveys are shown in *Figure 1.4*. Each plane has two observers for each transect who record the number of kangaroos seen. A correction factor is applied to the number of kangaroos sighted to account for the influence that vegetation, weather conditions and animal behaviour have on the ability to observe kangaroos from the air (Pople and Grigg 1999). The influence of past and future rainfall predictions on kangaroo populations are also taken into consideration (Department of Conservation and Land Management 2002). Population estimates are then calculated based on latest broad scale survey results and corrected using conservative adjustment factors (Department of Environment and Conservation 2008).

Extensive surveys of the *South Western Zone* were undertaken in 1981, 1984 and 1987 only. This was because the dense foliage associated with conservation land within the zone resulted in poor visibility. In 2004, surveys were reinitiated in the *South Western Zone* to improve the accuracy of population estimation. Aerial surveys of *Monitor Blocks* are now conducted annually (*Figure 1.4*) (Department of Environment and Conservation 2007; 2008).

1.4.4. Kangaroo Population Trends

The estimated WGK population in the *Central*, *South Western* and *South Eastern Zones,* from 1981 until present, are shown in *Figure 1.5* (Department of the Environment and Water Resources 2007). The WGK is not present in the *Northern Management Zone* of WA. Populations have remained above 1,400,000 animals over the past four years, peaking in 2007 at 1,893,295. Total population estimates are not available for 1987 – 2004 because aerial surveys were not undertaken in the *South Western Monitoring Zone* during this time.

The estimated total population of red kangaroos in the *Northern*, *Central* and *South Eastern Zones* from 1981 to present are shown in *Figure 1.6* (Department of the Environment and Water Resources 2007). The red kangaroo does not occur in the *South Western Zone*. Populations have fluctuated since 1981, but have declined over the past seven years. In 2006, red kangaroo numbers reached a ten-year low, at just over 1,000,000 animals. Population estimates were not reported for all *Management Regions* in 1995, 1996 and 1997 because of the transition from triennial surveys to annual surveys.

Figure 1.4 Population monitoring zones and flight lines used to survey kangaroo populations in Western Australia

(Department of Environment and Conservation 2008)

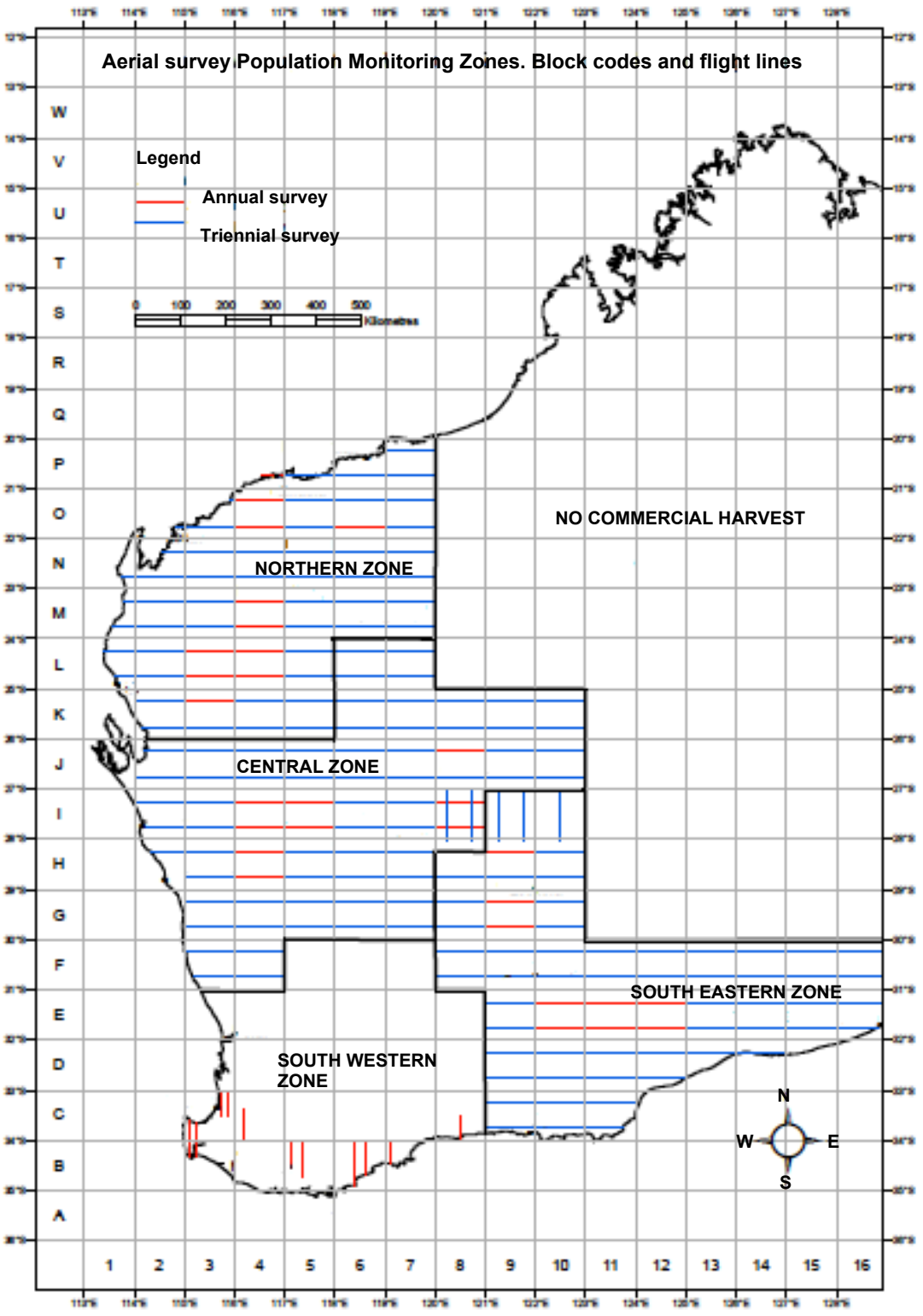


Figure 1.5 Annual population estimates of the western grey kangaroo (*M. fuliginosus)* in Western Australia

(Department of Environment and Conservation 2008)

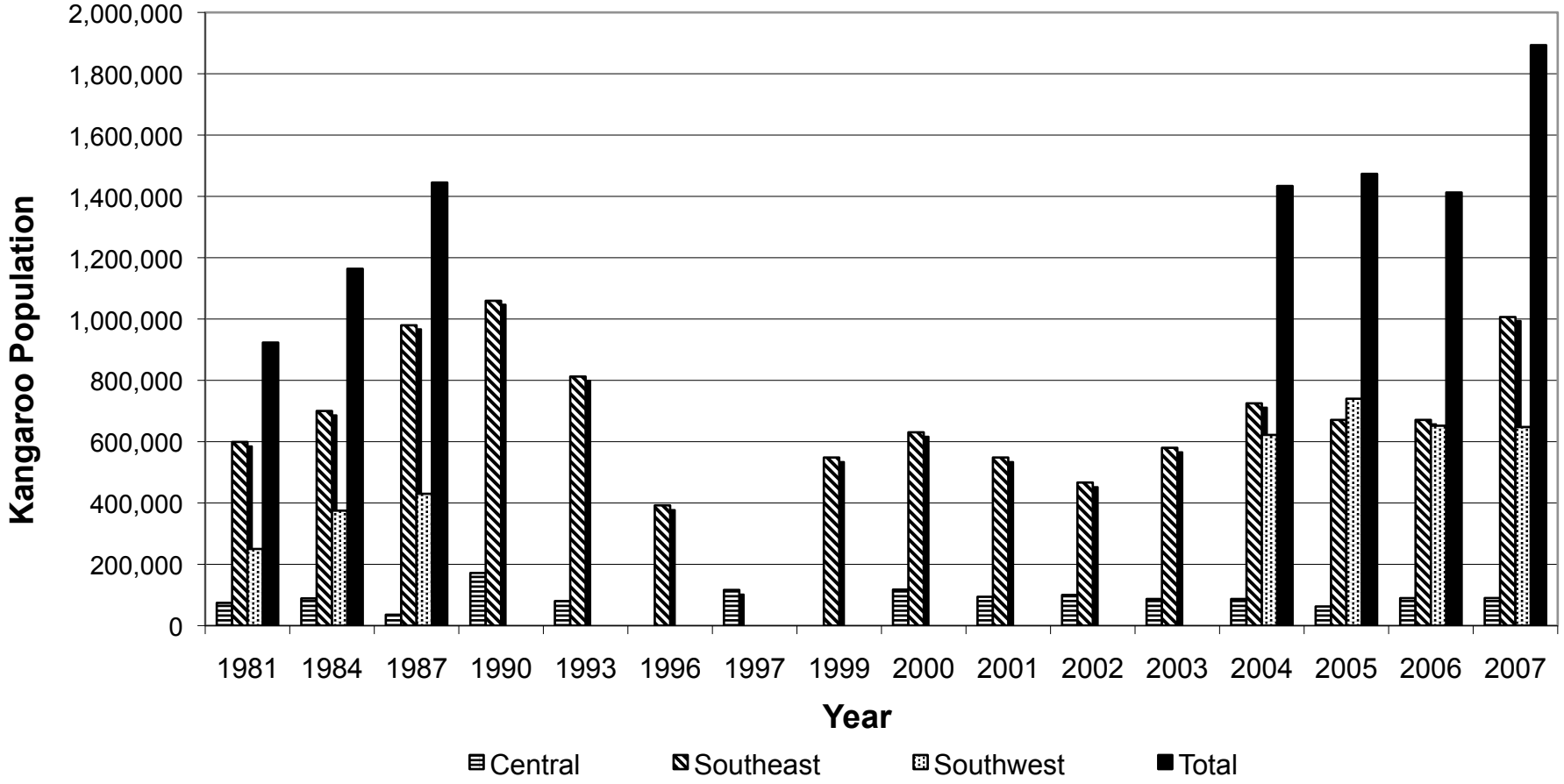
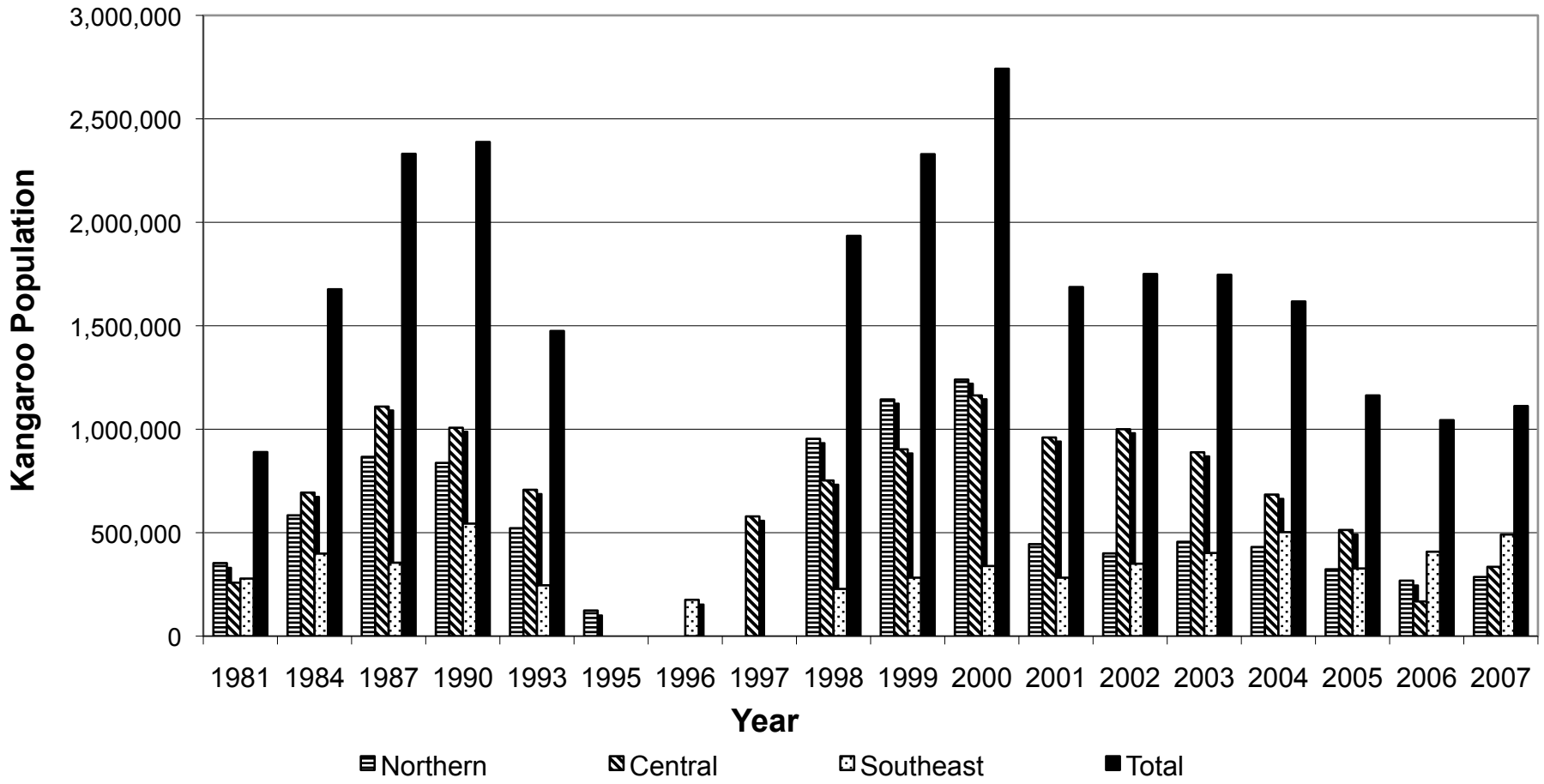


Figure 1.6 Annual population estimates of the red kangaroo (*M. rufus)* in Western Australia

(Department of Environment and Conservation 2008)



1.4.5. Quota Setting

Annual quotas are set to dictate the maximum number of kangaroos permitted for harvesting in the forthcoming calendar year (Department of Environment and Conservation 2008). The quota is established based on population estimates from the previous year together with a number of other factors including seasonal conditions, previous harvest returns, trends in land use, reports of damage to primary production, other forms of mortality apart from harvesting and extent of non-commercial culling (Department of Conservation and Land Management 2005; Department of the Environment and Heritage 2007). Prior to the commencement of each calendar year, draft quotas are set by DEC and the Kangaroo Management Advisory Committee (KMAC) and submitted to the Federal Minister for approval. Whilst quotas are set at a state level, each *Population Monitoring Zone* is allocated a notional sub-quota to prevent over-harvesting (Department of Conservation and Land Management 2002). As a guide, the annual quota for WGK is set at 12-15% of the total estimated population and the quota for red kangaroos is set at a maximum of 20% of the total population (Pople and Grigg 1999; Department of Environment and Conservation 2008). *Table 1.2* lists the annual quota for both the red and WGK over the past 17 years.

Table 1.2 Annual quotas and harvest data for the red and western grey kangaroo (1992 – 2007)

(Department of the Environment Water Heritage and the Arts 2007; Department of Environment and Conservation 2008)

Year	WGK Quota	Total WGK Harvested	% of Annual Quota	Red Quota	Total Reds Harvested	% of Annual Quota
1992	65, 000	45, 821	70.5	350,000	105,728	30.2
1993	65, 000	45,405	69.8	350,000	137,627	39.3
1994	60, 000	50,825	84.7	220,000	151,997	69.1
1995	60, 000	61,125	101.9	220,000	105,414	47.9
1996	80, 000	63, 478	79.4	160,000	126,084	78.8
1997	70, 000	50, 046	71.5	180,000	122,341	68.0
1998	74, 000	45, 674	61.7	180,000	116,727	64.8
1999	74, 000	58, 769	79.4	350,000	147,441	42.1
2000	104, 000	69, 553	66.9	350,000	124,866	35.7
2001	112, 000	87, 073	77.7	350,000	151,947	43.4
2002	95, 000	97, 074	102.2	250,000	221,596	88.6
2003	85, 000	99, 944	117.6	263,000	224,171	85.2
2004	121, 000	105, 308	87.0	262,000	232,562	88.8
2005	180, 000	158, 210	87.9	250,000	200,266	80.1
2006	193, 800	170,690	88.1	174,495	106,885	61.2
2007	197,780	126,309	63.9	126,585	119,094	94.1

1.4.6. Monitoring of Harvest Returns

Each professional shooter is required to complete a detailed report of the number of kangaroos shot, the location of harvest, the breakdown of males to females and their carcass weights for each night of harvesting. The Department of Environment and Conservation monitors kangaroo harvest figures every month through licensee logs to ensure the sustainability of the kangaroo harvesting industry (Department of Environment and Conservation 2008).

For the purpose of harvest monitoring, the four major *Population Monitoring Zones* of Western Australia are divided into 23 *Management Regions (Figure 1.7)*. Each *Management Region* is further divided into a series of smaller, more uniformly sized *Management Blocks,* delineated by equally spaced lines of latitude and longitude *(Figure 1.8)*. Each of the 77 blocks in the *South Western Zone* has been further divided into four *(Figure 1.9)*. These management divisions have been established to enable collation and analysis of harvest statistics on a geographically smaller scale.

The number of harvested WGKs increased significantly from 36,820 in 1990 to 126,309 in 2007 *(Table 1.2)*. During this time, an average of 83% (76.2, 89.7) of the annual commercial quota for the WGK was harvested each year. The number of harvested red kangaroos declined from 223,140 in 1990 to 119,094 in 2007, averaging 64.3% (55.0, 73.7) of the annual harvest quota *(Table 1.2)*. The individual harvest figures for each of the *Management Regions* in Western Australia between 1990 and 2006 are listed in *Table 1.3*. Harvesting was greatest overall in the Gascoyne and the Nullabor, followed by the Murchison, but limited in Bay Pastoral, Yilgarn and the

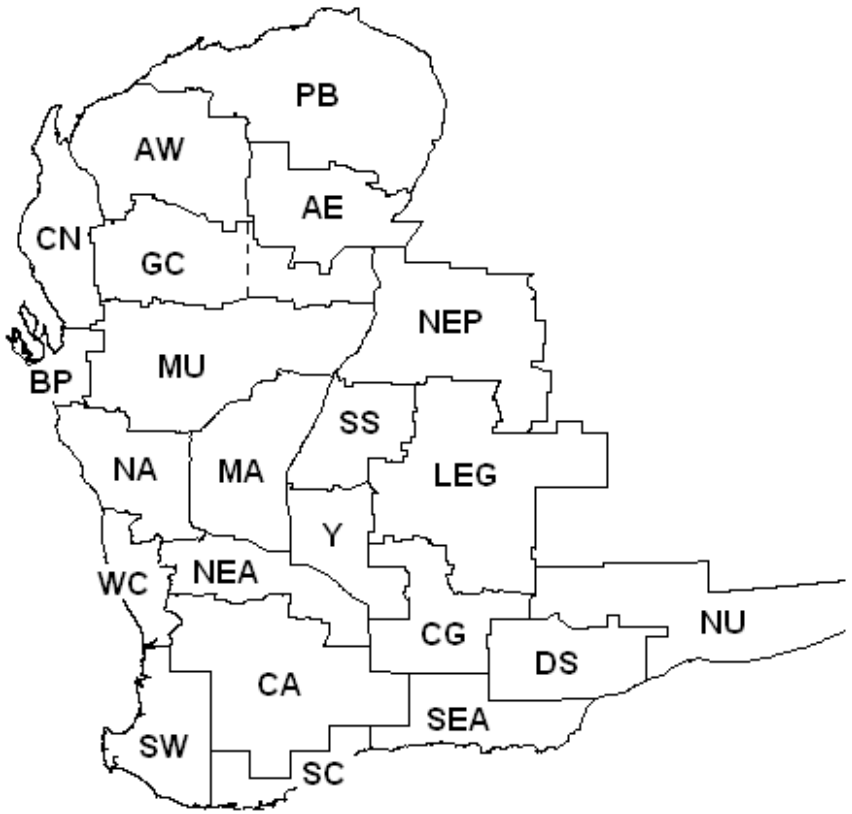
North Eastern Agricultural *Management Regions*. Red and WGKs were not harvested in a number of regions because their distribution did not extend that far.

1.4.7. Mandatory Tagging of Kangaroos

It is mandatory for shooters to tag each kangaroo carcass to assist in the management of the harvesting industry (Pople and Grigg 1999). Shooters are required to purchase royalty tags assigned a unique indentifying number from DEC (Department of Environment and Conservation 2008). The unique number is recorded against the shooter who purchased them, to prevent shooters from exchanging tags and to ensure carcasses can be traced back to the individual responsible for its harvest. Once the animal is eviscerated in the field, the shooter must place the tag through the skin just beneath the tail and adjacent to the rectum (Department of Environment and Heritage 1990). It is illegal for kangaroo carcasses or skins to be bought, sold or transported without an official tag attached (Department of Environment and Conservation 2008). Shooters of kangaroos for human consumption are also required by processors to attach a tag to each carcass with their name, the date and the location in which the kangaroo was shot (Frank Zambonetti, King River International, Personal Communication, April 24th, 2009).

Figure 1.7 Geographical breakdown of Western Australia into management regions for kangaroo harvest monitoring

(Department of Environment and Conservation, 2008)



Zone	Abbreviation	Management Region
Northern	AE	Ashburton East
	AW	Ashburton West
	PB	Pilbara
	CN	Carnarvon
	GC	Gascoyne (west)
Central	MU	Murchison
	NEP	North-East Pastoral
	GC	Gascoyne (east)
	MA	Magnet
	NA	Northern Agricultural
	BP	Bay Pastoral
	WC	Western Coastal
	SS	Sandstone
	Y	Yilgarn
South Eastern	LEG	Leonora-Eastern Goldfields
	CG	Coolgardie
	DS	Dundas
	NU	Nullarbor
	NEA	North Eastern Agricultural
	SEA	South Eastern Agricultural
	CA	Central Agricultural
	SC	South Coastal
South Western	SW	South West

Figure 1.8 Geographic breakdown of Western Australia into management blocks for harvest monitoring

(Department of Environment and Conservation 2008)

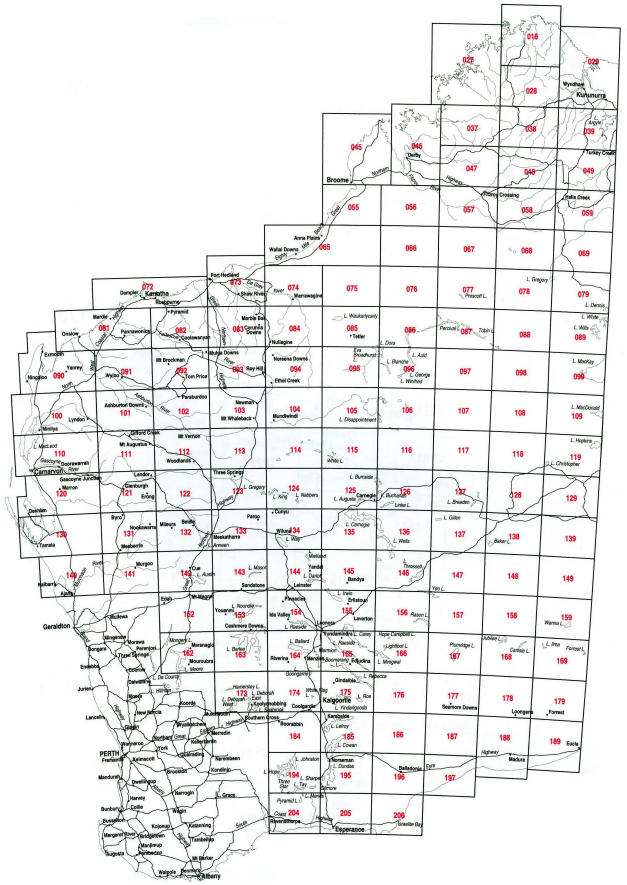


Figure 1.9 Geographic breakdown of the South Western management zone into management blocks for harvest monitoring

(Department of Environment and Conservation 2008)

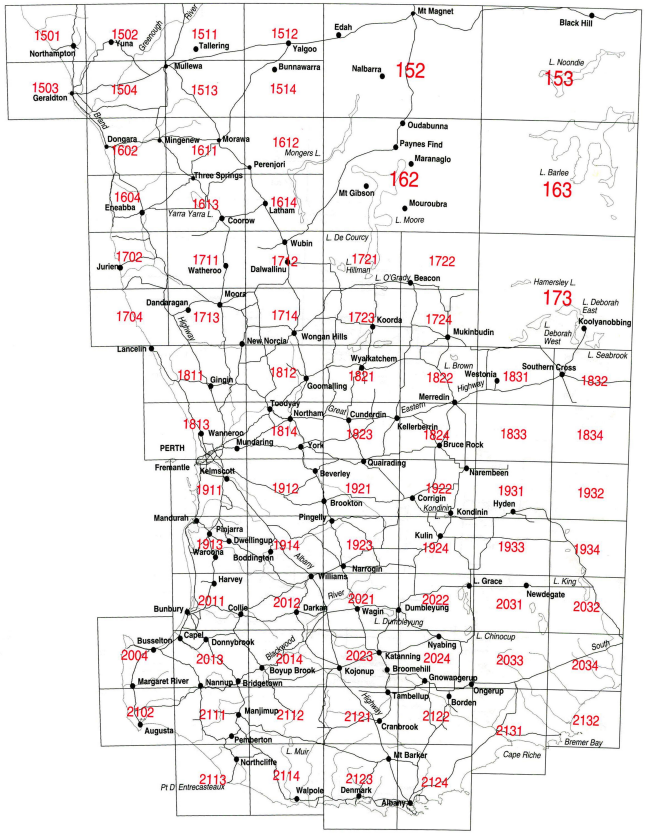


Table 1.3 Average number of red and western grey kangaroos harvested in each management region (1990 – 2006)

(Department of Environment and Conservation 2008)

Management Region	WGK Harvest	WGK (95% CI)	Red Harvest	Red (95% CI)	Combined Harvest	Combined (95% CI)
Ashburton East	0	0,0	2,015	1,016, 3,014	2,015	1,016, 3,014
Ashburton West	0	0,0	13,654	11,007, 16,301	13,654	11,007, 16,301
Bay Pastoral	37	16,59	48	0, 113	85	0, 156
Central Agricultural	5,533	4,181, 6,884	60	7, 113	5,593	4,242, 6,944
Carnarvon	0	0,0	11,166	9,139, 13,193	11,166	9,139, 13,193
Coolgardie	1,405	986, 1,824	2,317	1,717, 2,917	3,722	2,756, 4,688
Dundas	7,402	2,413, 12,391	1,334	520, 2,149	8,736	3,263, 14,210
Gascoyne	0	0,0	36,092	30,277, 41,907	36,092	30,277, 41,907
Leonora - East. Goldfields	3,099	2,339, 3,859	16,443	12,295, 20,590	19,542	14,984, 24,100
Magnet	84	46, 123	3,605	2,438, 4,773	3,690	2,502, 4,878
Murchison	133	85, 181	22,834	17,259, 28,409	22,966	17,385, 28,548

Table 1.3 cont. Average number of red and western grey kangaroos harvested in each management region (1990 – 2006)

(Department of Environment and Conservation 2008)

Management Region	WGK Harvest	WGK (95% CI)	Red Harvest	Red (95% CI)	Combined Harvest	Combined (95% CI)
Northern Agric.	3,756	2,689, 4,824	3,196	2,402, 3,991	6,953	5,396, 8,509
North East Agric.	451	244, 658	272	105, 440	723	368, 1,079
North East Pastoral	3	0, 6	6,851	3,391, 10,311	6,854	3,395, 10,313
Nullarbor	15,564	12,378, 18,750	23,036	16,893, 29,179	38,600	30,007, 47,194
Pilbara	0	0,0	8,941	6,560, 11,322	8,941	6,560, 11,322
South Coastal	13,048	10,861, 15,236	0	0, 0	13,048	10,861, 15,236
South East Agric.	2,676	963, 4,389	1	0, 4	2,677	964, 4,391
Sandstone	2	0, 5	5,931	3,693, 8,169	5,933	3,695, 8,172
South West	15,418	10,966, 19,871	1	0, 3	15,419	10,967, 19,872
West Coastal	6,829	4,234, 9,423	15	0, 32	6,843	4,243, 9,443
Yilgarn	21	0, 52	25	0, 53	47	0, 103

1.4.8. Harvest Activity of Professional Kangaroo Shooters in Western Australia

From 2001 to 2006, the top 10 per cent of professional shooters were responsible for removing an average of 52.2% (47.9 – 56.4) of the annual harvested population (*Table 1.4*). On average, only 227 (180 – 274) of the 312 (266 – 358) licences granted over this period were considered active. This suggests that fewer than 30 individuals were responsible for removing approximately half of the total harvest yield. Furthermore, three shooters were responsible for removing the top tenth percentile, averaging 12,397 animals each per year (*Figure 1.10)* (Department of Environment and Conservation 2008).

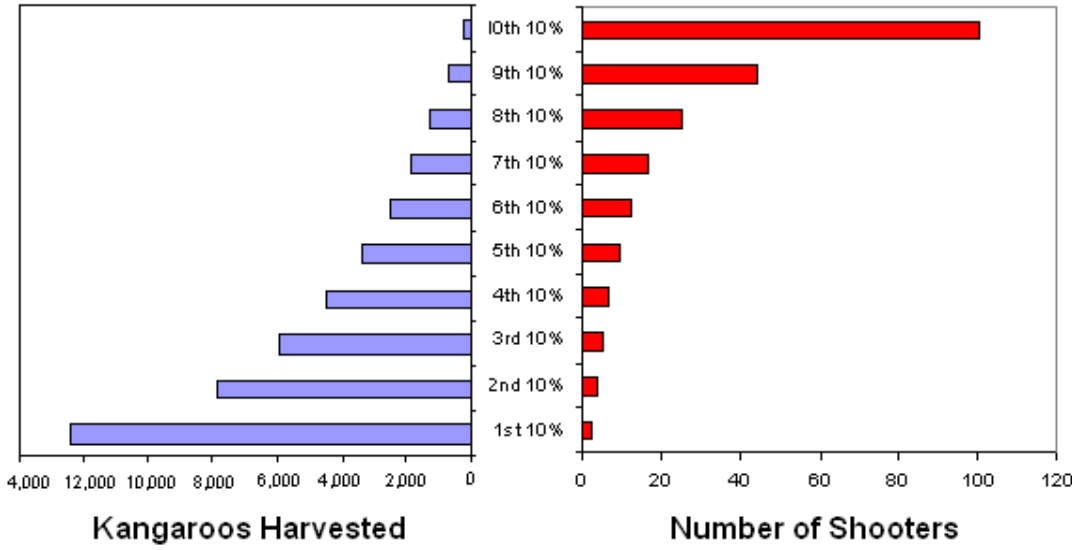
Table 1.4 Proportion of total harvest yield taken by top ten per cent of professional shooters in Western Australia

(Department of Environment and Conservation 2008)

Year	Proportion of Harvest Taken by Top 10% of Shooters
2001	52.0%
2002	52.0%
2003	49.0%
2004	45.0%
2005	61.0%
2006	54.0%

Figure 1.10 Number of shooters responsible for each 10th percentile of the annual harvest yield (2001 – 2006)

(Department of Environment and Conservation 2008)



1.5. Disease in Macropods

Clinical disease has been most commonly reported in kangaroos kept in captivity (Speare, Donovan et al. 1989). This is possibly because the stressors associated with capture, a change in diet and cohabitation with animals not normally encountered in close proximity causes the immune system to function at a less than optimal level (Arundel 1981). The most common pathogens to cause morbidity in kangaroos in their natural habitat include protozoan and metazoan parasites, but these rarely result in death (Speare, Donovan et al. 1989). Despite this finding, there have been a number of mass mortality events in the wild affecting kangaroo populations. In 1998, approximately 250,000 red kangaroos and 50,000 grey kangaroos were found dead over a period of 2 weeks in western NSW (Curran, Gay et al. 1999). The deaths occurred after good rain and adequate feed availability. Most animals died in good body condition. Similar 'epidemics' also occurred in Queensland in 1990 following heavy rains (Clancy, Southwell et al. 1991) and again in 1999 (Curran, Gay et al. 1999). The cause was never identified. Although death from disease in kangaroos may be the end point, often a combination of environmental factors such as drought, flood, habitat destruction or a feed shortage acts as the initiating stressor (Speare, Donovan et al. 1989).

The microorganisms and parasites that have been found to infect macropods are detailed in *Table 1.5*. Metazoan parasites were omitted because there were too many to list. The remainder of *Section 1.5* is dedicated to discussing *Salmonella*, *Coxiella burnetii* and Ross River virus in the kangaroo, as these three organisms are the focus of this research.

Table 1.5 List of microorganisms and parasites found to infect macropods

Infectious Agent	Disease	Citations
Bacteria		
Bartonella australis	Bartonellosis	(Fournier, Taylor et al. 2007; Woods 2008)
Coxiella burnetii	Q-fever *(unlikely to cause disease)*	(Derrick, Smith et al. 1939; Smith and Derrick 1939; Derrick, Smith et al. 1940; Pope, Scott et al. 1960; Arundel 1981; Beveridge 1981)
Clostridium tetani	Tetanus	(Speare, Donovan et al. 1989)
Fusobacterium necrophorum	Lumpy Jaw or Necrobacillosis	(Tomlinson and Gooding 1954; Arundel, Barker et al. 1977; Horton and Samuel 1978; Wilson, Taylor et al. 1980; Arundel 1981; Samuel 1983; Smith, Turner et al. 1986; Blanden, Lewis et al. 1987; Gulland, Lewis et al. 1987; Speare, Donovan et al. 1989; Dawson 2002)
Mycobacterium spp.	Mycobacteriosis	(Kennedy, Montali et al. 1978; Peet, Dickson et al. 1982; Speare, Donovan et al. 1989; Canfield and Hartley 1992; Young, McFarlane et al. 2003)
Salmonella spp.	Salmonellosis	(Winter 1957; Suzuki, Kawanishi et al. 1967; Iveson and Bradshaw 1973; Arundel 1981; Samuel 1982; Hart, Bradshaw et al. 1985; Speare and Thomas 1988; Bensink J.C., Ekaputra I et al. 1991 ; Hall and Rowe 1992; Eglezos, Sofroni et al. 2007; Holds, Pointon et al. 2008)
Viruses		
Wallal virus & Warrego virus	Kangaroo Blindness	(Blacksell, Lunt et al. ; Hooper 1999; Hooper, Lunt et al. 1999; Reddacliff, Kirkland et al. 1999)
Herpes virus	Herpes	(Webber and Whalley 1978; Acland 1981; Arundel 1981; Callinan and Kefford 1981; Kerr, Whalley et al. 1981; Speare, Donovan et al. 1989; Guliani, Smith et al. 1999)

Table 1.7 cont. List of microorganisms and parasites found to infect macropods

Infectious Agent	Disease	Citations
Ross River virus and Murray Valley encephalitis virus	*Unlikely to cause disease*.	(Marshall and Miles 1984; Kay, Young et al. 1985; Kay, Hall et al. 1986; Aldred, Campbell et al. 1991; Vale, Spratt et al. 1991; Lindsay 1995; Harley, Sleigh et al. 2001; Russell 2002; Old and Deane 2005; Oliveira, Broom et al. 2006; Leighton, Roitberg et al. 2008)
Macropod Pox virus	Pox virus	(McKenzie, Fay et al. 1978; Rothwell, Keep et al. 1984; Speare, Donovan et al. 1989)
Picornavirus	Foot and Mouth Disease	(Bhattacharya, Banerjee et al. 2003)
Fungi		
Dermatophytes	Ringworm	(Speare, Donovan et al. 1989; Staker 2006)
Protozoa		
Eimeria, Isospora, Klossiella, Sarcocystis.	Coccidiosis	(Winter 1959; Mykytowycz 1963; Calaby and Poole 1971; Barker, Harrigan et al. 1972; Finnie 1974; Arundel, Barker et al. 1977; Arundel 1981; Speare, Donovan et al. 1989)
Leishmania spp.	Leishmaniasis	(Rose 2004; Rose, Curtis et al. 2004)
Toxoplasma gondii	Toxoplasmosis	(Dobos-Kovacs, Meszaros et al. 1974; Jakob-Hoff and Dunsmore 1983; Obendorf and Munday 1983; Patton, Johnson et al. 1986; Dubey, Ott-Joslin et al. 1988; Johnson, Roberts et al. 1988; Johnson, Roberts et al. 1989; Canfield, Hartley et al. 1990; Miller, Ehlers et al. 1992; Reddacliff, Hartley et al. 1993; Gardner, Hietala et al. 1996; Turni and Smales 2001; Twomey, Higgins et al. 2002; Miller, Faulkner et al. 2003; Adkesson M.J., Gorman M.E. et al. 2007; Basso, Venturini et al. 2007; Dubey, Crutchley et al. 2008)

1.5.1. *Salmonella*

Salmonella nomenclature is complex and evolving (Brenner, Villar et al. 2000). The genus *Salmonella* currently consists of only two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies, which are referred to by a Roman numeral and a name (Brenner, Villar et al. 2000; OIE 2008):

- I *Salmonella enterica* subsp. *enterica*
- II *Salmonella enterica* subsp. *salamae*
- IIIa *Salmonella enterica* subsp. *arizonae*
- IIIb *Salmonella enterica* subsp. *diarizonae*
- IV *Salmonella enterica* subsp. *houtenae*
- VI *Salmonella enterica* subsp. *indica*

The individual names of each serotype in subspecies I continue to be used for identification, whilst antigenic formulae are cited for unnamed serotypes described after 1966 within the remaining subspecies and in *S. bongori*. On the first citation of a serotype from subspecies I, the genus name is given followed by the word "serotype" or the abbreviation "ser.", then the serotype name. Subsequently, the name may be written with the genus followed directly by the serotype name. The serotype name is usually capitalised and not italicised (Brenner, Villar et al. 2000), although variations of this exist in the literature.

Macropods can harbour *Salmonella* in their gastrointestinal tracts and lymph nodes (Samuel 1982). Whilst kangaroos can carry and shed *Salmonella* with no clinical signs, it has also been reported to cause death as a primary pathogen and an opportunistic invader of the intestinal tract (Samuel 1982). Salmonellosis is the

clinical manifestation of infection (Blood and Studdert 1999), causing varying levels of gastroenteritis and septicaemia in hand-reared joeys and animals housed in captivity (Speare, Donovan et al. 1989).

1.5.1.1. Evidence of *Salmonella* in Live Kangaroos

Salmonella was first isolated from kangaroos in the 1950's (Winter 1957), but macropods were only suspected of carrying high levels of the organism following infection in two cats fed kangaroo meat in the 1960's (Anderson, Crowder et al. 1964). Consumption of pet meat preparations containing kangaroo was subsequently considered a risk factor for *Salmonella* infection in pets (Anderson, Crowder et al. 1964). As pet food is often stored in domestic refrigerators and prepared using kitchen utensils, contaminated kangaroo meat may also be a potential source of *Salmonella* for people living in the household. Suzuki et al. (1967) reported contamination rates as high as 44.9% in kangaroo meat imported into Japan for human consumption (Suzuki, Kawanishi et al. 1967).

Following a case of salmonellosis in a young infant on Rottnest Island, *Salmonella* spp. were isolated from 71% of quokkas (*Sentonix brachyurus*) on Rottnest Island (Iveson and Bradshaw 1973). The prevalence of infection peaked as high as 70 – 100% in summer when feed quality declined and digestive physiology was disrupted and dropped as low as 0 – 30% in winter when feed quality improved (Hart, Bradshaw et al. 1985). Whilst the quokka and kangaroo are both free-ranging macropods, care must be taken in extrapolating these findings to mainland animals. The population of quokkas studied live in a closed population and are exposed to

unusually high levels of contact with humans and their waste products, increasing the risk of infection (Samuel 1982).

The prevalence of *Salmonella* in captive-reared kangaroos is also particularly high. Fifty one percent of kangaroos tested in Queensland were shown to be infected with *Salmonella* (n=90) (Arundel 1981). Seventy three of these animals were born and reared in captivity, 11 were rescued as joeys and kept as pets and only six were wild animals (Arundel 1981). Thomas et al. (2001) isolated *Salmonella* spp. on 62 occasions from 57 macropds over a period of 20 years from mostly captive-reared kangaroos. Thirty five isolates were cultured from faecal samples alone, 14 were from lymph nodes and the remainder were from urine, liver, lung, kidney and spleen samples. Speare and Thomas (1988) also found that 26.8% of orphaned young were infected with 16 different serotypes of *Salmonella* spp. and 21.7% were actively excreting the bacterium in faeces (Speare and Thomas 1988).

The most recent review of *Salmonella* in wildlife identified 85 different serotypes associated with Macropodoididae, only three of which were from the WGK and nine from the red kangaroo (Speare, Donovan et al. 1989). It is reasonable to assume that the spectrum of isolates residing in the gastrointestinal tract of kangaroos is far greater than those formally published, given that more than 2500 serotypes have been isolated globally (Health Protection Agency Laboratory of Enteric Pathogens 2007).

1.5.1.2. *Salmonella* Contamination of Kangaroo Meat

The presence of *Salmonella* in kangaroo meat sold in supermarkets suggests that infection and shedding of the organism occurs in free-ranging macropods (Samuel

1982). In the first major study of *Salmonella* contamination in kangaroo meat intended for human consumption within Australia, 11.1% of muscle samples excised from carcasses in a processing plant were culture-positive (Bensink, Ekaputra et al. 1991). In a more extensive investigation of 836 carcasses from two kangaroo processing plants in Queensland, only 0.84% of muscle samples excised from carcasses contained *Salmonella* (Eglezos, Sofroni et al. 2007). Subsequent surveys of kangaroo carcasses and meat have shown a low prevalence of contamination of the outer surface of carcasses (~1%) and higher rates of contamination of abdominal cavities and mince meat (12-18%) (Holds, Pointon et al. 2008). The increased number of positive abdominal swabs reflects the increased risk of contamination of this area resulting from gut perforation during evisceration. Common serotypes isolated in the above studies include *Salmonella* serovars Muenchen, Chester, Havana, Rubislaw and Singapore. Other isolates include *Salmonella* serovars Orion, Senftenberg, Emmastad, Eastbourne, Saintpaul, Reading, Zehlendorf, Infantis, Fremantle, Anatum, Sofia and Kottbus (Bensink, Ekaputra et al. 1991; Eglezos, Sofroni et al. 2007; Holds, Pointon et al. 2008).

1.5.1.3. National Enteric Pathogens Surveillance Scheme

The National Enteric Pathogens Surveillance Scheme (NEPSS) has collected, analysed and disseminated data on enteric infections diagnosed in Australia since 1980 (Department of Health and Ageing 2008). *Salmonella*-positive samples have been obtained from swabs and specimen samples taken from the stomach, small intestine, large intestine, rectum, lymph nodes, spleen, lung, liver, kidney and urine of kangaroos during this period. The aggregated data from NEPSS shows that 23.7% of isolations were from wild kangaroos and the remainder were from animals kept as

pets or in captivity. The three most commonly isolated *Salmonella* serotypes from both live kangaroos and kangaroo meat were *Salmonella* serovars Muenchen, Typhimurium and Chester. All reports of *Salmonella* in red and grey kangaroos and kangaroo meat collated by the NEPSS between 1981 and 2006 are listed below in *Table 1.6* and *Table 1.7*, respectively. Whilst there have been no published reports of contaminated kangaroo meat causing salmonellosis, a number of the serotypes isolated from kangaroos and their meat products by the NEPSS have been known to cause food-borne disease in people. In 2006, *S. enterica* ser. Typhimurium was the most commonly reported serovar causing infection in Australians (OzFoodNet Working Group 2007). Phage type 135 was the most prolific subtype and this was isolated from a grey kangaroo in Victoria and eight samples of kangaroo meat during the reporting period (National Enteric Pathogen Surveillance System 2007). In Western Australia, *Salmonella* serovars Saintpaul and Muenchen were among the top five serotypes isolated from individuals suffering from salmonellosis (OzFoodNet Working Group 2007). *Salmonella* serovars Chester, Havana, Senftenberg, Singapore, Anatum, Kiambu and Infantis have also been associated with food-borne salmonellosis in recent years (OzFoodNet Working Group 2002; 2005; 2006; 2007).

Despite the high prevalence of *Salmonella* in animals in captivity and contamination of kangaroo meat for pet and human consumption, no study has been undertaken to determine the prevalence of naturally acquired infection in animals in the wild. Until such a study is carried out it is not possible to comment on whether kangaroos naturally harbour larger quantities of *Salmonella* than common domestic livestock species.

Table 1.6 *Salmonella* spp. isolated from live kangaroos in Australia (1981 – 2006)

Salmonella Serotype	Status	State Isolated	No. Isolates
S. ser. Adelaide	C	NT, WA	3
S. ser. Agona	C	QLD	1
S. ser. Alsterdorf subsp II	U	WA	1
S. ser. Anatum	W, U	QLD, SA, VIC, WA	6
S. ser. Bahrenfeld	U	WA	1
S. ser. Birkenhead	P	QLD	1
S. ser. Bootle	U	WA	1
S. ser. Bovismorbificans (14, 31, 32)	U, C, W, P	QLD, TAS, NSW, QLD	7
S. ser. Chester	C, P, W	WA, QLD, SA, NT, ACT	28
S. ser. Eastbourne	W	NSW	2
S. ser. Enteritidis (14, RDNC/01)	W	QLD	2
S. ser. Fremantle subsp II	W, C	SA, VIC, WA	6
S. ser. Give	W, C	QLD, SA, WA, VIC	7
S. ser. Havana	C, W	NSW, SA, WA, QLD	10
S. ser. Heidelberg	P	QLD	1
S. ser. Hessarek (var 27+)	U	VIC	1
S. ser. Infantis	C, W	QLD, NSW, VIC	3
S. ser. Kiambu	C	WA	1
S. ser. Kinondoni	C	NSW, QLD	2
S. ser. Kottbus	C, P, U	QLD, SA, VIC,	12
S. ser. Lansing	U, W	QLD, WA	2
S. ser. Litchfield	C	QLD	1
S. ser. Mbandaka	W, C	QLD	5

C Captivity W Wild

P Pet U Unknown

Table 1.8 cont. *Salmonella* spp. isolated from live kangaroos in Australia (1981 – 2006)

Salmonella Serotype	Status	State Isolated	No. Isolates
S. ser. Muenchen	C, U, W	QLD, VIC, WA, SA, NSW, NT	24
S. ser. Newport	W	WA	1
S. ser. Oranienburg	C, U	WA, SA	4
S. ser. Orientalis	C, P	QLD, SA	3
S. ser. Orion	C, U, W	QLD, WA, NSW	4
S. ser. Potsdam	C	QLD	1
S. ser. Rubislaw	W	WA	2
S. ser. Saintpaul	C, P, W, U	SA, VIC, QLD, WA	8
S. ser. Schwarzengrund	U	VIC	1
S. ser. Singapore	C, W	NSW, VIC, WA	5
S. ser. Sofia subsp II	U	VIC	1
S. ser. Stanley	C	VIC	1
S. ser. Tennessee	C, W	NSW, VIC	2
S. ser. Typhimurium (4, 9, 12a, 22, 44, 108, 135, 145, 176, 177, RDNC, U307)	C, U, W	NSW, WA, SA, WA, VIC, QLD	31
S. ser. Victoria	C	VIC	1
S ser. Virchow	P	QLD	2
S. ser. Wandsbek subsp II	C, W	SA, WA	3
S. ser. Waycross	C	QLD, SA, NSW	6
S. ser. Zanzibar	C, P	NT, QLD	2
S. subsp IIIb (41:z4,z23:-, 48:k:z53, 50:k:z35)	C, W, P	QLD, VIC	3

C Captvity W Wild

P Pet U Unknown

Table 1.7 *Salmonella* spp. isolated from kangaroo meat in Australia (1981 – 2006)

Salmonella Serotype	State Isolated	No. Isolates
S. ser. Adelaide	NSW, VIC, SA, WA	14
S. ser. Anatum	VIC, QLD, SA, WA	10
S. ser. Bahrenfeld	QLD, SA	7
S. ser. Bergedorf	NSW	1
S. ser. Bovismorbificans	VIC, WA	3
S. ser. Bredeney	WA	1
S ser. Bukavu	WA	1
S. ser. Cerro	QLD	2
S. ser. Champaign	VIC	3
S. ser. Charity	SA, WA	3
S. ser. Chester	VIC, QLD, SA, WA	63
S. ser. Derby	WA	1
S. ser. Eastbourne	WA	1
S. ser. Fremantle subsp. *II*	NSW, VIC, QLD, SA, WA	39
S. ser. Give	VIC, QLD, WA	12
S. ser. Havana	VIC, QLD, SA, WA	19
S. ser. Hvittingfoss	WA	1
S. ser. Infantis	QLD	3
S. ser. Jangwani	WA	1
S. ser. Kottbus	NSW, VIC, QLD, SA, WA	21
S. ser. Liverpool	WA	1
S. ser. Livingstone	WA	1
S. ser. Mbandaka	WA	1
S. ser. Muenchen	NSW, VIC, QLD, SA, WA	60
S. ser. Muenster	SA	1
S. ser. Ohlstedt	QLD	2
S. ser. Onderstepoort	QLD, SA	2
S. ser. Oranienburg	NSW, VIC, QLD, WA	7
S. ser. Orientalis	VIC, WA	7
S. ser. Orion	SA, VIC, WA	7

Table 1.7 cont. *Salmonella* spp. isolated from kangaroo meat in Australia (1981 – 2006)

Salmonella Serotype	State Isolated	No. Isolates
S. ser. Orion var 15+	SA	1
S. ser. Potsdam	QLD, SA, VIC, WA	10
S. ser. Reading	QLD	2
S. ser. Rubislaw	QLD, VIC, WA	6
S. ser. Saintpaul	QLD, SA, WA	8
S. ser. Senftenberg	WA	1
S. ser. Singapore	SA, VIC, WA	8
S. ser. Sofia subsp II	SA, WA	3
S. ser. Tennessee	QLD, SA, WA	15
S. ser. Thompson	WA	1
S. ser. Typhimurium	WA	1
S. ser. Typhimurium 101	NSW	1
S. ser. Typhimurium 135	QLD, NSW	8
S. ser. Typhimurium 156	WA	1
S. ser. Typhimurium 170	VIC	2
S. ser. Typhimurium 22	QLD	1
S. ser. Typhimurium RDNC	QLD	42
S. ser. Urbana	WA	3
S. ser. Wandsbek subsp II	QLD, VIC, WA	4
S. ser. Wandsworth	WA	3
S. ser. Welikade	QLD, WA	7
S. ser. Zehlendorf	QLD, SA	2
S. subsp. I ser. 1,4,5,12:-:-	WA	1
S. subsp. I ser. 40:l,z28:-	QLD	1
S. subsp I ser rough:b:1,5	VIC	1
S.. subsp. II ser. 16:g,m,t:-	SA	1
S. subsp. III (not typed)	WA	1
S. subsp. IIIb 48:r:z	SA	1
S. subsp. IIIb 61:z52:z53	SA	1

1.5.2. *Coxiella burnetii*

Q fever is a zoonotic disease caused by the Gram-negative intracellular bacterium, *Coxiella burnetii* (Beveridge 1981; Arricau-Bouvery and Rodolakis 2005). Human infection takes on one of three forms; asymptomatic (60%), acute (flu-like, pneumonia, hepatitis) and chronic (endocarditis, post-Q fever fatigue syndrome) (Arricau-Bouvery and Rodolakis 2005). The disease occurs world-wide with the exception of Antarctica and possibly New Zealand (Hilbink, Penrose et al. 1993; Greenslade, Beasley et al. 2003) and is now considered a re-emerging zoonosis in many countries (Arricau-Bouvery and Rodolakis 2005). Currently, *C. burnetii* is classed as a Category B bioterrorism threat by the Centers for Disease Control and Prevention in the USA (CDC 2008).

Livestock have traditionally been associated with transmission of *C. burnetii* with the majority of infections occurring in agricultural workers involved with the farming or slaughter of ruminants (Garner, Longbottom et al. 1997). Infections in sheep, cattle and goats can cause abortion, stillbirth, retained placenta, endometritis, infertility and small or weak offspring (Arricau-Bouvery and Rodolakis 2005). Indirect infection can also occur through inhalation of the organism in stockyards or drinking non-pasteurised milk (Beveridge 1981). The organism can survive for long periods of time in dust, sheep wool, dried faeces and milk (Speare, Donovan et al. 1989). A vaccine, Q-VAX, was developed by CSL and is very effective at providing immunity in people against Q-fever infection (Department of Health and Ageing and National Health and Medical Research Council 2008). A blood and intradermal skin test are required prior to administration of the vaccine to exclude those likely to have hypersensitivity reactions from previous exposure. It is recommended that abattoir workers, farmers,

stockyard workers, shearers, animal transporters and all others exposed to cattle, sheep, goats and kangaroos or their products are vaccinated. This includes veterinarians and laboratory personnel who are likely to work in environments where the organism may be present (Australian Technical Advisory Group on Immunisation 2007; Department of Environment and Conservation 2007). A vaccination program for cattle in Slovakia in the 1970's and 1980's was successful in reducing the occurrence of Q fever but the approach was not widely adopted due to the cost and technical factors involved in vaccine production (Arricau-Bouvery and Rodolakis 2005). More recently, due to a significant and ongoing outbreak of Q fever in the Netherlands, mandatory vaccination of small ruminants in high-incidence regions has been enforced to reduce the number of cases of human disease (Van der Hoek, Dijkstra et al. 2010).

1.5.2.1. *Coxiella burnetii* in Kangaroos

Derrick et al. (1939) proposed that marsupials were a possible reservoir host of *Coxiella burnetii* because of the results from experimental infection of bandicoots. Bandicoots were susceptible to infection and developed antibodies, although they did not exhibit clinical signs (Derrick, Smith et al. 1939). Pope et al. (1960) subsequently found that 18% of macropods tested (n=270) were positive for *C. burnetii* complement-fixing antibodies, agglutinating antibodies, or both. Red kangaroos had a higher prevalence of complement fixing antibodies (33%) compared to grey kangaroos (12%). Isolation of the organism was also achieved from one animal, implying that systemic infection does occur in kangaroos. Seropositive kangaroos were found in eleven of the fourteen districts investigated in Queensland, suggesting that exposure to the organism was widespread (Pope, Scott et al. 1960). *Coxiella burnetii* was also isolated from 13 kangaroo ticks (*Amblyomma triguttatum*),

four of which were found on goats and sheep. *Amblyomma triguttatum* is a 3-host tick and thus may be able to act as a vector between the different host species (Pope, Scott et al. 1960). The potential role of the WGK in the maintenance and transmission of *C. burnetii* was highlighted in a recent study where 33.5% (n=343) of animals were positive for *C. burnetii* antibodies using an ELISA (Banazis 2009; Banazis, Bestall et al. 2010). This was the first study investigating the role of macropods in the transmission of *C. burnetii* since 1960 and employed testing methods considered to be more sensitive than those adopted by Pope et al. (1960). Given the close association between kangaroos and grazing livestock in rural areas, together with exposure to arthropod vectors, the role of the kangaroo in transmission of *C. burnetii* warrants further investigation. It is important to characterise the relationship between domestic and wildlife cycles of *C. burnetii* in Australia. Research in this area may provide valuable information relating to the zoonotic threat that marsupials pose as a reservoir for human outbreaks of Q fever.

1.5.3. Ross River Virus

Ross River virus (RRV) is classified within the genus Alphavirus in the family Togaviridae (Harley, Sleigh et al. 2001; Smith, Mackenzie et al. 2008). As transmission between vertebrate hosts is **ar**thropod **bo**rne, RRV is known as an **arbo**virus (Marshall and Miles 1984; Kay and Aaskov 1989; Lindsay 1995; Harley, Sleigh et al. 2001; Russell 2002; Lindsay 2004). The clinical manifestation of infection is known as Ross River virus disease. The single-stranded, positive-sense RNA virus (Harley, Sleigh et al. 2001) was first isolated from mosquitoes in 1963 (Doherty, Whitehead et al. 1963) and later, from a human suffering from epidemic polyarthritis (Doherty, Carley et al. 1972). The incubation period usually lasts 7 to 9

days but can vary between 3 to 21 days (Harley, Sleigh et al. 2001; Smith, Mackenzie et al. 2008). Symptoms include fatigue, fever, myalgia, headache and rash (Marshall and Miles 1984; Kay and Aaskov 1989; Harley, Sleigh et al. 2001; Smith, Mackenzie et al. 2008). Whilst many individuals remain asymptomatic, those that do become clinically affected may display self-limiting symptoms that persist for approximately four weeks. A chronic course of symptoms may persist in affected individuals for months to years (Smith, Mackenzie et al. 2008). Diagnosis is ideally made through a combination of identification of characteristic clinical signs, recent history of mosquito exposure and serological evidence of recent infection. IgM serology alone is not definitive because RRV-specific IgM can persist for months after infection (Smith, Mackenzie et al. 2008). Paired blood tests, taken 10-14 days apart, showing greater than a four fold increase in IgG, is generally considered a positive result (Smith, Mackenzie et al. 2008).

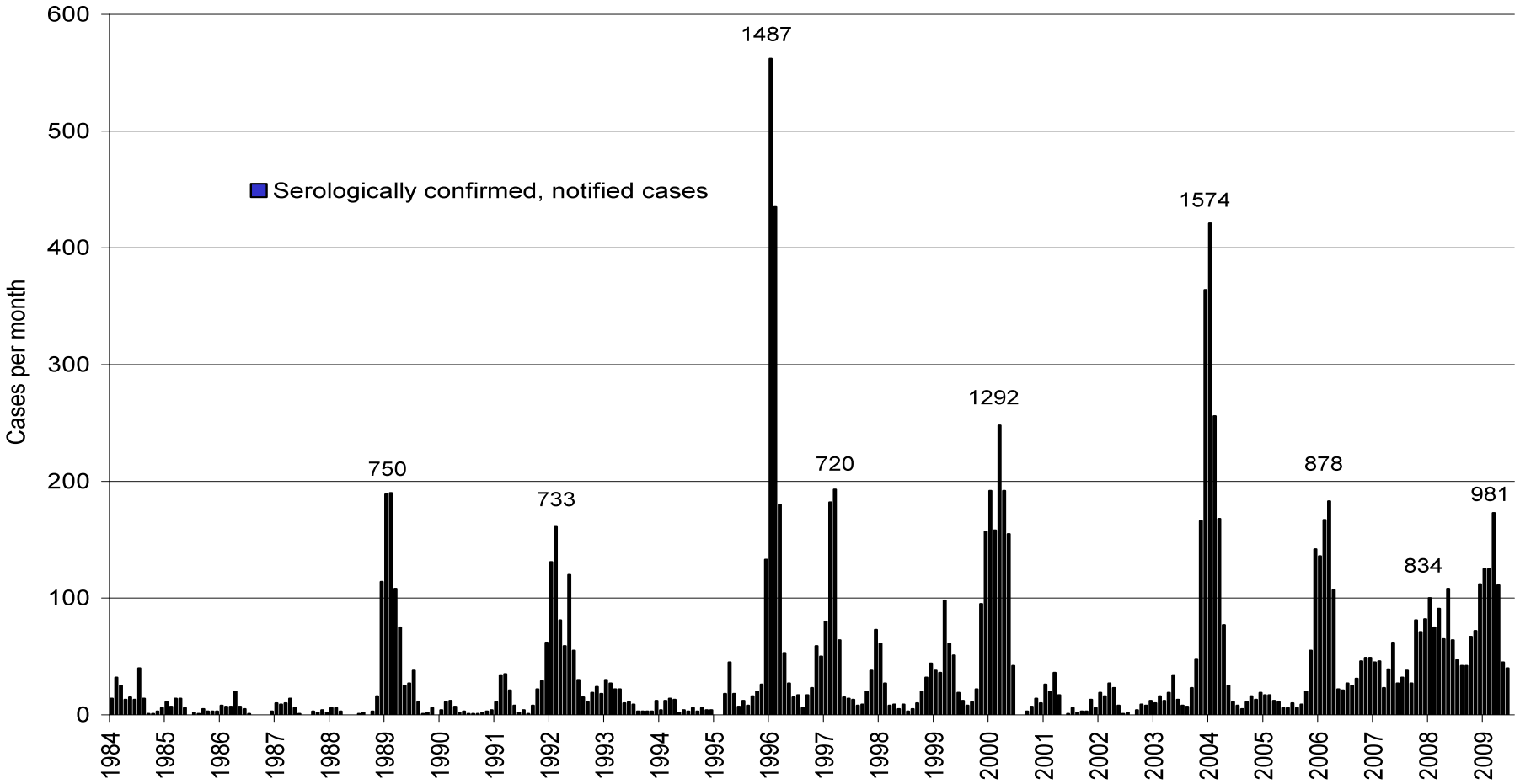
1.5.3.1. Clinical Incidence of Ross River Virus in Western Australia

Ross River virus (RRV) disease is the most common mosquito-borne disease both nationally and in Western Australia (Lindsay, Oliveira et al. 1997; Done, Holbrook et al. 2002; Lindsay 2004; Russell and Kay 2004; Gatton, Kay et al. 2005). As it is a notifiable disease, all serologically confirmed cases must be reported to the WA Department of Health by general practitioners or the State diagnostic laboratory PathWest (Lindsay, Oliveira et al. 1997). Local Government Environmental Health Officers will then conduct a follow-up investigation to ascertain the most likely place and timing of exposure of the individual to potentially infected mosquitoes (Environmental Health Directorate 2006). The Mosquito-Borne Disease Control

Branch (MBDC) of the WA Department of Health monitors all doctor-notified and laboratory reported cases of RRV disease (Lindsay, Breeze et al. 2005).

The monthly incidence of serologically confirmed cases of RRV disease reported to the WA Department of Health between 1984 and 2009 is shown in *Figure 1.11* (Mosquito Borne Disease Control Branch 2009). A distinct cyclic trend in significant outbreaks of clinical disease within the state's human population is evident. A small number of human cases of RRV disease are reported every year in the Peel, Leschenault and Capel-Busselton localities, however, large outbreaks occur every three to four years (Lindsay, Oliveira et al. 1996; Johansen, Broom et al. 2005; Lindsay, Breeze et al. 2005). The inter-outbreak period probably occurs because of a combination of suboptimal climatic conditions, insufficient mosquito populations and/or insufficient numbers of susceptible vertebrate hosts (Johansen, Broom et al. 2005; Lindsay, Breeze et al. 2005).

Figure 1.11 Monthly incidence of Ross River virus disease in people in Western Australia (1984 – June 2009)



The magnitude and timing of RRV transmission in WA varies dramatically from region to region due to seasonal and annual variation in environmental conditions. Higher rainfall, warmer temperatures and tidal patterns in coastal regions of WA generally create favourable conditions for mosquito breeding in late winter, through spring to early summer (August-December). The first cases of human disease are reported in September, peaking in January and tapering off from April to May (Lindsay, Oliveira et al. 1997; Lindsay, Breeze et al. 2005). Western Australia's two largest recorded outbreaks of Ross River virus disease were experienced in the 1995/96 and 2003/04 seasons (Lindsay, Oliveira et al. 1997; Lindsay, Breeze et al. 2005). In 1995/96, the highest attack rates were noted in Augusta-Margaret River and Donnybrook-Balingup, followed by the Capel-Busselton and Leschenault regions (Lindsay, Oliveira et al. 1997). In 2003/04, more than 82% of cases reported were acquired in the southwest of the state, with the majority occurring from Mandurah to Dunsborough (Lindsay, Breeze et al. 2005). Clusters of cases of RRV disease have also been reported in other regions of Western Australia, particularly in the southern outskirts of Perth and the Southwest region.

In northern Western Australia, RRV disease is considered to be endemic in nature, with outbreaks tending to coincide with the wet season, January – April (Lindsay 1995; 2004; Lindsay, Breeze et al. 2005). The incidence of RRV disease is more variable in the Pilbara, Gascoyne, Murchison and Goldfields, as a result of inconsistent rainfall. Epidemics are more characteristic in these areas with the maximum number of clinical cases reported between April and June. The risk of infection in the North Coastal and North Central districts is considered low compared

to districts further north and in the southwest of Western Australia, with small numbers of clinical cases occurring evenly throughout the year (Lindsay 1995).

1.5.3.2. Transmission of Ross River Virus

Ross River virus is maintained within the environment through transmission between competent mosquito vectors and susceptible vertebrate hosts. Those arthropods capable of acting as vectors do so most commonly through ingestion of a viraemic blood meal, followed by viral amplification and finally secretion of the virus in the saliva prior to feeding on a susceptible vertebrate host (Liehne 1991). Only the female mosquito serves as a vector of RRV (Liehne 1991; Lindsay and Mackenzie 1997). Trans-ovarial transmission of Ross River virus is also possible, resulting in vertical transmission through the reproductive cycle (Kay and Aaskov 1989; Vale, Dowling et al. 1992). This may be important for viral persistence in regions where mosquito populations fluctuate due to seasonal variation (Lindsay, Broom et al. 1993). Whilst humans were once considered incidental hosts of the virus, a number of outbreaks in Australia and Fiji suggest otherwise. Under unusual circumstances, it is though that a viraemic individual may act as an initial source of infection in a naïve population and possibly become involved in a mosquito-human-mosquito transmission cycle (Aaskov, Mataika et al. 1981; Rosen, Gubler et al. 1981; Marshall and Miles 1984; Sammels, Coelen et al. 1995).

1.5.3.3. Vectors of Ross River Virus in Western Australia

In Western Australia, the most common mosquitoes capable of transmitting RRV include species from the genera *Aedes* and *Culex* (Lindsay and Mackenzie 1997). Three vector species principally transmit RRV; *Culex annulirostris*, *Aedes vigilax* and

Aedes camptorhynchus (Harley, Sleigh et al. 2001; Russell 2002). Other genera that may also be involved in RRV transmission include *Anopheles, Mansonia, Coquillettidia, Culiseta* and *Tripteroides* (Lindsay 1995). The most likely vectors for the study areas from which kangaroos were sampled are listed in *Table 1.8.*

1.5.3.4. Mosquito Surveillance in Predicting Ross River Virus Activity

The Arbovirus Surveillance and Research Laboratory (ASRL), in collaboration with the WA Department of Health, undertake regular mosquito trapping along the Swan Coastal Plain in the southwest of Western Australia all year round. The primary aim of this surveillance is to monitor mosquito populations throughout the year, identify species in abundance and detect viral activity. Prior to and during the peak arboviral season (September – April), fortnightly EVS (Encephalitis Vector Survey) traps, using carbon dioxide as the primary attractant, are set at sunset and collected again the following morning at sunrise (Johansen, Broom et al. 2005). Between May and August, mosquitoes are still collected but virus isolation is not performed (Personal Communication, Cheryl Johansen, ASRL, December 2008). Mosquitoes are then transported back to the laboratory where a representative sample are identified and processed for virus isolation (Lindsay, Oliveira et al. 1997).

Data from mosquito surveillance was successfully used to predict both large RRV epidemics in the south-coastal region of WA over the 1995/96 and 2003/04 seasons (Lindsay, Oliveira et al. 1997; Johansen, Broom et al. 2005; Lindsay, Breeze et al. 2005; Johansen, Broom et al. In Press). Combining results from mosquito surveillance with rainfall, tide and temperature data, can be very useful in improving the capacity to predict viral activity (Lindsay 2004; Woodruff, Guest et al. 2006).

Table 1.8 Suspected/confirmed mosquito vectors of Ross River virus in development regions of Western Australia relevant to sample collection locations

District	Species	Vector Status
South Coastal	*Aedes camptorhynchus*	**Confirmed Vector** (Ballard 1982; Marshall and Miles 1984; Ballard and Marshall 1986; Lindsay, Oliveira et al. 1997; Russell 2002; Johansen, Broom et al. 2005)
	Aedes vigilax (Summer Saltmarsh Mosquito)	**Confirmed Vector** (Ballard 1982; Kay 1982; Vale, Dowling et al. 1992; Wells, Russell et al. 1993; Lindsay, Oliveira et al. 1997; Ryan and Kay 1997; Ryan, Do et al. 2000; Kay and Jennings 2002)
	Aedes notoscriptus (Container Mosquito)	**Confirmed Vector** (Ballard 1982; Doggett and Russell 1997; Watson and Kay 1998; Ryan, Do et al. 2000; Russell 2002)
	Culex annulirostris (Common Banded Mosquito)	**Confirmed Vector** (Ballard 1982; Marshall and Miles 1984; Liehne 1991; Wells, Russell et al. 1993; Ryan, Do et al. 2000; Russell 2002)
	Aedes clelandi	**Suspected Vector** (Lindsay 2004)

Table 1.8 cont. Suspected/confirmed mosquito vectors of Ross River virus in developmet regions of Western Australia relevant to sample collection locations

Region	Species	Vector Status
Metropolitan Perth	*Aedes notoscriptus*	**Confirmed Vector** (as above).
	Aedes vigilax	**Confirmed Vector** (as above).
	Culex annulirostris	**Confirmed Vector** (as above).
	Aedes camptorhynchus	**Confirmed Vector** (as above).
	Coquillettidia spp. near *linealis*	**Suspected Vector** (Marshall and Miles 1984; Russell 2002; Smith, Mackenzie et al. 2008)
Midwest & Wheatbelt	*Aedes sagax*	**Suspected Vector** (Liehne 1991; Russell 2002; Smith, Mackenzie et al. 2008)
	Aedes vigilax	**Confirmed Vector** (as above).
	Aedes camptorhynchus	**Confirmed Vector** (as above).
	Aedes notoscriptus	**Confirmed Vector** (as above).
	Culex annulirostris	**Confirmed Vector** (as above).

1.5.3.5. Environmental Factors Determining Ross River Virus Activity

Mosquito populations are dramatically affected by climatic and environmental variables such as rainfall, temperature, humidity, wind, atmospheric pressure and tidal patterns (Lindsay and Mackenzie 1997; Tong and Hu 2001; Done, Holbrook et al. 2002; Kelly-Hope, Purdie et al. 2004; Tong, Hu et al. 2005; Lindsay 2006). These variables influence mosquito breeding, development, survival, host-seeking behaviour and a range of other biological traits (Lindsay and Mackenzie 1997). The presence of water is vital for at least one stage of all mosquito breeding (Mellor and Leake 2000; Russell 2006). Temperatures influence the rate of development and survival of mosquito larvae and adults, and many species experience temperature restricted breeding (Weinstein 1997; Lindsay 2006). Humidity is particularly important for adult mosquito survival, dispersal, mating, feeding and oviposition (Mellor and Leake 2000; Tong and Hu 2001).

A large number of retrospective studies have been undertaken to correlate climatic variables with mosquito surveillance data and the incidence of RRV disease in people, to improve prediction of viral activity. Tong and Hu (2001) combined notified RRV disease case data with climate and population data in Cairns. They identified a significant positive correlation between RRV disease incidence and current maximum temperature, rainfall and humidity at a lag phase of two months (Tong and Hu 2001). Other studies have demonstrated correlation between the disease incidence and the Southern Oscillation Index, La Nina and the Quasi-Biennial Oscillation (Harley and Weinstein 1996; Done, Holbrook et al. 2002; Kelly-Hope, Purdie et al. 2004). Kelly-Hope et al. (2004) noted that whilst climatic and tidal factors acted differently in tropical, arid and temperate zones, average rainfall appeared to be the single most

important risk factor in activity of RRV. Gatton et al. (2005) also noted that no one set of climatic variables could be applied to all regions to predict RRV activity. However, warmer temperatures and increased rainfall were the two most important factors in all studies (Gatton, Kay et al. 2005). Woodruff et al. (2006) undertook an extensive study in the southwest of Western Australia to evaluate the use of combining mosquitoes and climate surveillance data. On its own, climatic surveillance data had a sensitivity of 64% and a specificity of 96% for predicting RRV epidemics (Woodruff, Guest et al. 2002). The sensitivity of the model was increased to 90% when mosquito surveillance data was included (Woodruff, Guest et al. 2002). Woodruff et al. (2006) found that climatic data recorded later than November did not increase the sensitivity of the model, suggesting that climatic prerequisites for an impending epidemic are already established by this time (Woodruff, Guest et al. 2006). In the southwest of WA, abnormally high tides and late rainfall favour mosquito breeding, often resulting in large populations of *Aedes camptorhynchus* and *Aedes* v*igilax* in spring and summer (Mackenzie, Lindsay et al. 2000; Kelly-Hope, Purdie et al. 2004). This is a significant risk factor for a large outbreak of RRV, particularly in the Peel, Leschenault and Capel-Busselton regions which experience the highest attack rates in the southwest (Lindsay 2004).

1.5.3.6. Vertebrate Hosts of Ross River Virus

The vertebrate host species involved in RRV transmission have not yet been confirmed (Harley, Sleigh et al. 2001). Key steps to defining a vertebrate host include:

1. Determining if the animal is susceptible to infection and capable of developing a viraemia of sufficient titre and duration to infect competent vectors (infection studies);

2. Determining if the animal is fed on by competent local vector mosquito species (vector bloodmeal analysis) and
3. Demonstrating that the animal is naturally infected in the wild (serosurveys).

1.5.3.6.1. Ross River Virus Isolation from Potential Vertebrate Hosts

Ross River virus has only been isolated from non-human vertebrate hosts on 7 occasions. These include two horses, two agile wallabies (*Macropus agilis*) and three birds of varying species (Whitehead, Doherty et al. 1968; Doherty, Standfast et al. 1971; Pascoe, St George et al. 1978; Harley, Sleigh et al. 2001). Ross River virus is difficult to isolate because of the short viraemic period and lack of clinical signs in non-human vertebrate hosts. It was first isolated from the heart muscle of three birds at Mitchell River Mission in April 1965 (Whitehead, Doherty et al. 1968). Since this time, there has been no further evidence to suggest that birds play a significant role in transmission (Marshall and Miles 1984). The virus was later isolated from two agile wallabies in this same area, during which time, 88% of the 147 wallabies tested for antibodies were also seropositive (Doherty, Standfast et al. 1971). In April 1978, RRV was successfully isolated from the blood of an apparently healthy, eight year old mare (Pascoe, St George et al. 1978). At the time, the animal was negative for neutralising antibodies, but developed an antibody titre within the following two months (Pascoe, St George et al. 1978).

1.5.3.6.2. Experimental Infection of Potential Vertebrate Hosts wth Ross River Virus

Early experimental infection of vertebrates with RRV demonstrated that rabbits, rats, marsupial mice, bandicoots and day old chickens were capable of producing a

viraemia (Whitehead 1969). Over the next two decades, successful experimental infection studies expanded to include a range of domestic livestock, birds and marsupials. Most significantly, the eastern grey kangaroo and agile wallaby were capable of mounting a viraemia following infection (Kay, Hall et al. 1986).

The role of horses and flying foxes in transmission of RRV continues to remains unclear. Kay et al. (1987) noted that whilst only one of the eleven horses infected with RRV developed a viraemia detectable by inoculation of suckling mice, a total of five were capable of reinfecting mosquitoes. Similarly, flying foxes (*Pteropus poliocephalus*) were unable to produce a viraemia of sufficient magnitude to be detected by conventional RRV assay techniques, however virus was recovered from feeding *Ae. Vigilax.* This indicated that *P. poliocephalus* were capable of developing a viraemia and reinfecting mosquitoes (Ryan, Martin et al. 1997). There is evidence to suggest that the common brushtail possum (*Trichosurus vulpecular*) is a potential reservoir host for RRV. Three of ten possums infected with RRV developed a detectable viraemia and at 24 hours post infection, 53% of mosquitoes feeding on the animals were also infected (Boyd, Hall et al. 2001). All three animals showed clinical signs within four days following infection, experiencing a combination of ataxia, lethargy, inappetence and increased recovery time from anaesthesia. The common brushtail possum is a ubiquitous urban marsupial in Australia and may play a role in the transmission of RRV in and around cities (Boyd, Hall et al. 2001). Infected dogs and cats failed to produce a detectable viraemia and no mosquitoes feeding on the animals became infected (Boyd and Kay 2002). Only one dog and one cat developed neutralising antibodies to RRV, suggesting that they are unlikely reservoirs of the virus (Boyd and Kay 2002). The results of the studies conducted to date suggest that marsupials are better amplifiers of RRV than mammals, which in turn, are better

than birds (Marshall and Miles 1984; Kay, Hall et al. 1986; Kay and Aaskov 1989; Harley, Sleigh et al. 2001).

1.5.3.6.3. Serological Evidence of Ross River Virus Infection in Potential Vertebrate Hosts

The detection of antibodies to RRV using serological tests provides evidence that an individual has been infected with the virus (Lindsay 1995). However, it is difficult to determine whether antibody development is indicative of a true amplifying host or whether the species is capable of developing antibodies without playing a role in viral transmission (Boyd and Kay 2002).

Serum neutralising antibodies to RRV have been found in a range of animals including horses, cattle, goats, sheep, pigs, camels, buffalo, possums, kangaroos, wallabies, small rodents, flying foxes, domestic cats and dogs and a small number of birds (Spradbrow 1972; Gard, Marshall et al. 1973; Rosen, Gubler et al. 1981; Cloonan, O'Neill et al. 1982; Marshall and Miles 1984; McManus and Marshall 1986; Kay and Aaskov 1989; Aldred, Campbell et al. 1991; Vale, Spratt et al. 1991; Lindsay 1995; Azuolas 1997; Harley, Sleigh et al. 2001; Old and Deane 2005; Kay, Boyd et al. 2007). A number of these animals developed antibodies following experimental infection (Whitehead 1969; Kay, Hall et al. 1986; Kay, Pollitt et al. 1987; Ryan, Martin et al. 1997; Boyd, Hall et al. 2001; Boyd and Kay 2002). Of those animals found to have neutralising antibodies, only laboratory mice/hamsters, horses (Kay and Aaskov 1989; Lindsay 1995; Azuolas 1997) and brushtail possums (Boyd, Hall et al. 2001) are suspected to exhibit clinical signs. Although based on limited experimental

infection studies, there is no evidence to suggest that kangaroos develop clinical signs following infection with RRV (Kay, Hall et al. 1986).

Antibody production was induced in an eastern grey kangaroo within seven days of inoculation with RRV (Kay, Hall et al. 1986). In a serological study of 39 eastern grey kangaroos in Victoria, 36% were seropositive to RRV (Aldred, Campbell et al. 1991). Vale et al. (1991) similarly found that macropods had the highest proportion of seropositivity (68%) of all animals tested. In eastern New South Wales, a total of 11% and 33% of two captive populations of Tammar wallabies (*Macropus eugenii*) were also seropositive for RRV antibodies (Old and Deane 2005). In WA, 35% of all WGKs sampled across a number of geographical regions were positive for RRV neutralising antibodies (Lindsay 1995).

1.5.3.6.4. Blood Meal Analysis in Mosquitoes

Analysis of the blood meal taken by mosquitoes known to be capable of transmitting RRV provides evidence that marsupials are commonly fed upon by these vectors (Leighton, Roitberg et al. 2008). In Western Australia, *Ae. vigilax, Ae. camptorhynchus* and *Cx. annulirostris* commonly feed on marsupials (Lindsay 1995; Johansen, Power et al. 2004; Johansen, Power et al. 2009). This finding is particularly significant as both *Ae. camptorhynchus* and *Cx. annulirostris* are considered important vector species in the southwest of Western Australia (Harley, Sleigh et al. 2001; Russell 2002).

1.5.3.6.5. Vertebrate Host Immunity

The marsupial immune response has many characteristics analogous to that of eutherian species. Four out of the five major immunoglobulin isotypes found in eutherians have now been identified in marsupials. Immunoglobulin M and IgG have been detected in a number of species, including the Virginian opossum (*Didelphis virginiana*), koala (*Phascolarctos cinereus*), brush-tail possum (*T. vulpecula*), quokka (*S. brachyurus*), hill kangaroo (*M. robustus*), eastern grey kangaroo (*M. giganteus*) and the tammar wallaby (*M.eugenii*) (Rowlands, Dudley et al. 1968; 1969; Bell, Lynch et al. 1974; Bell, Stephens et al. 1974; Wilkinson, Allanson et al. 1991; Ramadass and Moriarty 1992; Wilkinson, Kotlarski et al. 1994; Rawson, Belov et al. 2002; Daly, Digby et al. 2007). Immunoglobulin A has also been detected in the quokka, brushtail possum and tammar wallaby (Bell, Stephens et al. 1974; Ramadass and Moriarty 1992; Adamski and Demmer 1999; Daly, Digby et al. 2007). More recently, the genes encoding IgG, IgM, IgA and IgE have been isolated and sequenced in the tammar wallaby, brushtail possum and the grey short-tailed opossum (*Monodelphis domestica*) (Aveskogh and Hellman 1998; Belov, Duckworth et al. 1998; Adamski and Demmer 1999; Belov, Harrison et al. 1999; Daly, Digby et al. 2007). Despite these similarities, it is difficult to predict the likely immune response to RRV infection in macropods because few experimental infection studies have been undertaken. It is generally accepted that if a host has had previous exposure to a virus and maintains antibodies of sufficient magnitude, a second viraemia is not likely to develop (Carver, Bestall et al. 2008).

Marsupials are born in an altricial state of development with an immature immune system (Daly, Digby et al. 2007). Maternal transfer of immunity via milk increases

following the birth of the young and again during the switch phase, which is just prior to exit from the pouch (Bell, Stephens et al. 1974; Deane, Cooper et al. 1990; Adamski and Demmer 1999; Daly, Digby et al. 2007). These two periods of increased immune transfer coincide with times of increased immune challenge (Daly, Digby et al. 2007). Maternally acquired antibodies in the quokka wane within 4-6 weeks of their absence in milk, with a half-life of 8-9 days. Antibody titres in quokka pouch young have been found to be higher than the maternal serum, particularly at the switch phase (Yadav and Eadie 1973; Deakin and Cooper 2004). This is likely due to the ability for the young to mount its own immune response in preparation for increased antigenic challenges (Belov, Mai-Anh et al. 2002). Ross River virus antibodies have been detected in two kangaroo pouch young aged 3 and 6 weeks old, respectively (Lindsay 1995). As the joeys were not old enough to have been exposed to the external environment at this early stage, immunity is likely to have been acquired through passive transfer from the doe.

1.6. Conclusion

This Literature Review has provided an extensive overview of research undertaken in the area of disease surveillance in wildlife, with particular emphasis on *Salmonella*, *Coxiella burnetii* and RRV in macropods. These organisms were specifically chosen not just because they pose a significant zoonotic threat, but because they each utilise different modes of transmission amongst vertebrate hosts; including arthropod-borne, food-borne and environmental transmission mechanisms.

Following recognition of where further research is required, the following objectives for this thesis were defined:

1. To establish a framework for active disease surveillance in kangaroos using the commercial harvesting industry.
2. To determine the prevalence of *Salmonella* in free-ranging kangaroos across a range of geographical locations and over an extended period of time.
3. To determine the seroprevalence of *C. burnetii* antibodies and prevalence of faecal isolation of *C. burnetii* in free-grazing kangaroos in Western Australia.
4. To further define the role of the western grey kangaroo as a reservoir host of RRV and to assess whether surveillance in these animals could improve the accuracy of predictions of viral epidemics in human populations in Western Australia.

2. UTILISING THE KANGAROO HARVESTING INDUSTRY FOR DISEASE SURVEILLANCE

2.1. Introduction

Emerging infectious diseases (EIDs) are infections that have newly appeared in a population or have existed previously but are rapidly increasing in incidence or geographic range (Morse 1995). Wildlife species often play a significant role in the transmission of EIDs, serving as reservoir hosts. Active surveillance in wildlife is an effective way of detecting the presence of EIDs in these species, ensuring swift counter measures are initiated to minimise the risk of disease establishment and spread (Morner, Obendorf et al. 2002). Kangaroos are potentially significant reservoirs of a number of infectious organisms, including *Salmonella, Coxiella burnetii,* Ross River virus and *Toxoplasma*. More recently, they have been implicated in the transmission of *Leishmania* in the Northern Territory of Australia (Rose 2004; Rose, Curtis et al. 2004; Dougall, Shilton et al. 2009) and Foot and mouth disease (Bhattacharya, Banerjee et al. 2003) in India. Surveillance of free-living kangaroo populations may assist in reducing the transmission of disease to people and domestic livestock in Australia.

Kangaroos are abundant throughout Australia, interacting with humans and domestic livestock through the utilisation of common resources (Daszak, Cunningham et al. 2000). In the pastoral zones of Australia, the provision of artificial watering points and irrigated pastures has created a niche habitat for the kangaroo (Department of Environment and Conservation 2008). This has resulted in growing populations of kangaroos competing with and forming a close association with livestock.

Urbanisation and the shrinking size of wildlife habitats have also forced kangaroos into close proximity with people, increasing the risk of zoonotic disease transmission.

With the increasing awareness of the role wildlife species play in infectious disease maintenance and transmission, the kangaroo harvesting industry is a potentially valuable resource that could be utilised for surveillance purposes. In Western Australia alone, more than 200,000 red (*Macropus rufus)* and western grey (WGK) (*Macropus fuliginosus*) kangaroos are harvested annually. Nationwide, this total exceeds more than 3,000,000 animals (Department of the Environment Water Heritage and the Arts 2007).

2.1.1. Aims of the Study

The primary aim of this study was to determine the suitability of using samples collected via the kangaroo harvesting industry for active disease surveillance. Specifically, this involved determining the most practical and cost effective means of sample collection, storage and transport, tailored to the routine of professional shooters. It also involved evaluating potential sources of bias that may reduce the statistical validity of the data generated.

2.2. Materials and Methods

2.2.1. Recruitment of Professional Kangaroo Shooters

Forty professional shooters, holding a *Wildlife Conservation Regulation 6 Licence,* from specific geographic locations were invited by letter (*Appendix A*) to participate in this study (human ethics permit no. 2006/242). Each shooter was asked to complete a questionnaire (*Appendix* B) to ascertain their level of involvement with the

harvesting industry and their knowledge of infectious diseases in kangaroos. A pre-paid envelope was provided to maximise the return rate of questionnaires. A contact number was also provided if they preferred to respond via telephone. A follow-up phone call was made to each professional shooter who returned the questionnaire and requested more information, or expressed a willingness to participate. Each shooter was asked to collect blood from a minimum of 15 – 20 kangaroos each month from the same location for the duration of the study and record the approximate age group and sex of the animals. Four professional shooters assisted with sample collection during this study, identified as professional shooters A, B, C and D. Additional sample collection was undertaken opportunistically by accompanying a fifth shooter, E. A further six shooters had initially expressed their willingness to participate but ceased participation shortly after the study began.

Each kangaroo shooter was sent a polystyrene cool box containing a letter of introduction and thanks (*Appendix C*) as well as instructions for sample collection (*Appendix D*). Contained in the cool box were fifty 10ml screw cap, serum blood tubes with clot retraction beads (46.390.001, SARSTEDT Australia Pty Ltd, Australia), foam tube storage racks, indelible pens, plastic specimen storage bags (Hercules® and Glad® supermarket brands) and tags for animal identification. Each tag was a 5cm by 5cm square of plastic coated cardboard with a unique identification number written on it with indelible pen. A hole was punched through the tag to allow a thick elastic band or small cable tie to be threaded through for attachment to the animal. Once shooters received their sample collection kits, a final follow-up phone call was made to ensure each person understood the requirements of the study.

2.2.2. Sample Collection

2.2.2.1. Geographic Locations

Serum, faecal and diaphragm muscle samples were collected from WGKs harvested at fifteen different locations in Western Australia between May 2006 and May 2009. Sampling sites included Capel, Myalup, Preston Beach, Eneabba, Badgingarra, Manjimup, Nannup, Bridgetown, Northcliffe, Boyup Brook, Balingup, Scott River, Greenbushes, Thomsons Lake and Whiteman Park (*Figure 2.1*).

2.2.2.2. Animal Data

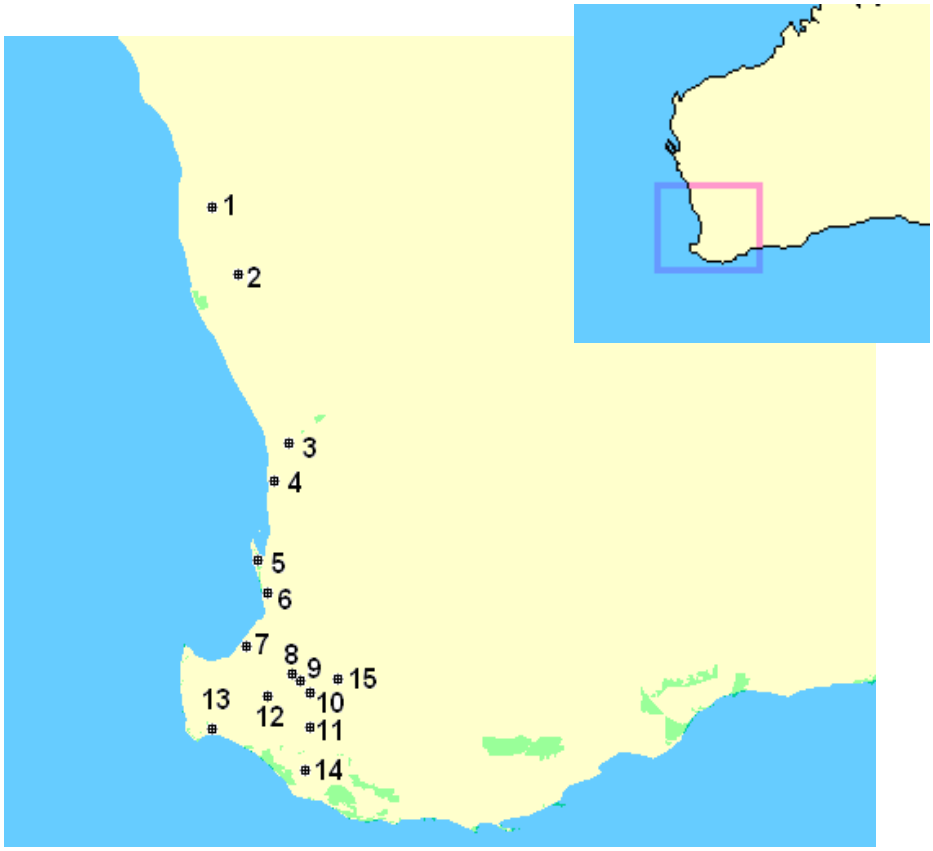
After kangaroos were confirmed deceased, a numbered tag was placed around each animal's hind leg for ease of identification. Blood was then collected and the tag number, sex and age of the animal were recorded on the serum and specimen collection bags. The age of the animal was determined subjectively based on the size of the animal and its apparent sexual maturity. Animals were considered adult (A) if they appeared fully grown and sexually mature (3 years and above), subadult (SA) if they were out of the pouch but not yet fully grown (< 3 years) and pouch young (P) if they were taken from the pouch to be sampled.

2.2.2.3. Harvest Data

Harvest data, including the number and sex of kangaroos harvested, for the commercial kangaroo industry in Western Australia was supplied by the Department of Environment and Conservation, WA.

Figure 2.1 Geographic locations of kangaroo sampling sites in Western Australia

(Generated using DiscoverAUS software (Magellan 2005))



Key

No.	Location	Shooter	No.	Location	Shooter
1	Eneabba	C	9	Greenbushes	B
2	Badgingarra	C	10	Bridgetown	B
3	Whiteman Park	E	11	Manjimup	B
4	Thomsons Lake	D	12	Nannup	B
5	Preston Beach	C	13	Scott River	B
6	Myalup	A	14	Northcliffe	B
7	Capel	A	15	Boyup Brook	B
8	Balingup	B			

2.2.2.4. Blood Sample Collection

Two collection methods were used to obtain blood samples from kangaroos; a free-catch technique used to collect blood directly into a 10ml serum tube with clot retraction beads (46.390.001, SARSTEDT Australia Pty Ltd, Australia) following incision of the carotid and jugular vessels and secondly, cardiac puncture using a sterile, 9 ml vacuette serum tube (Vacuette Serum Tube 455092, Greiner Bio-One, USA) and an 18 gauge, 1½ inch vacutainer needle (PrecisionGlide™, Becton Dickinson Vacutainer Systems, Plymouth, UK). For the cardiac puncture technique, the needle was inserted into the third rib space, perpendicular to the surface of the thorax, when the animal lying on the ground. If the animal was hanging, the needle was inserted into the second rib space because the heart moved in a cranial direction within the thorax. In smaller animals, the vacuum was activated once the needle had been inserted half way to avoid penetrating through the heart. In larger, male kangaroos the needle was inserted fully prior to activation of the vacuum. Gloves were worn to prevent the collector's hands becoming contaminated with blood. Samples were refrigerated at 4°C or stored in a cool box with an ice brick until serum was harvested, generally within 48 hours. Once a blood clot had retracted, disposable, non-sterile pasteur pipettes were used to harvest serum into smaller 2 ml free standing, screw top, serum tubes (Scientific Specialists Inc., USA). Serum was stored at -20°C until required for use.

2.2.2.5. Faecal Sample Collection

Faecal samples were collected as each kangaroo was being eviscerated in the field. With the abdominal organs externalised but still attached to the carcass, the distal colon was identified and a small number of faecal pellets were massaged caudally into the lower colon to ensure they remained following removal of the intestines. Evisceration was then completed and 10 cm of the caudal portion of gut containing the faecal sample was left *in situ*. The faecal pellets were then massaged from the intestines into a plastic specimen storage bag without coming into contact with the collector's hands. The samples were placed into a styrofoam cool box for the remainder of the harvest and were stored at 4°C for *Salmonella* culture or -20°C for *C. burnetii* PCR, within 24 hours of collection.

2.2.2.6. Diaphragm Muscle Sample Collection

Samples of diaphragm muscle were collected from kangaroos at two stages of the harvesting process, depending on the purpose of harvest (ie. pet food or human consumption). Samples were collected in the field from kangaroos harvested for pet meat because the thoracic and abdominal contents are removed shortly after the animal is shot. The remnants of the diaphragm's muscular periphery were excised from the thoracic wall by the shooter, placed in a plastic specimen storage bag and labelled with the animal's sex, age group and tag identification number. These specimens were considered "one-day old" diaphragm muscle samples and were stored at -20°C within 24 hours of collection. A total of 194 "one-day old" muscle samples were collected.

Seventy nine “one day old” samples were divided into two portions at the point of collection and placed into separate labelled specimen bags. The first sample was stored at -20°C while the second sample was stored at 4°C for ten days, prior to freezing (at -20°C), to approximate the storage time of a carcass in a chiller in the field and at the processing plant prior to processing. The latter specimens were referred to as “ten-day old” samples. Both one- and ten-day old samples were then stored at -20°C until required for meat juice extraction.

Sixty one diaphragm muscle samples were collected at the King River International processing plant in Canning Vale, Perth from stored kangaroo carcasses (up to ten days after harvest) intended for human consumption. Samples had to be collected from the processing plant because shooters for human consumption are required to leave the thoracic contents intact for inspection. Numbered identification tags were placed on each animal’s right leg by the shooter in the field using cable ties. The right leg remained on the animal until the last stage of processing to ensure identification was possible during sampling. Sampling was undertaken after the animal’s thoracic cavity was opened and its contents removed. Diaphragm muscle was excised from its attachment to the thoracic wall. Each sample was placed into a plastic specimen storage bag and numbered according to the identification tag attached to the animal’s leg. Samples were stored at -20°C until required.

2.2.2.7. Meat Juice Collection

Meat juice was obtained from diaphragm muscle using methods adapted from Nielsen et al. (1988). Briefly, samples were frozen in specimen storage bags for a minimum of 24 hours at -20°C and then thawed at room temperature. After thawing, meat juice

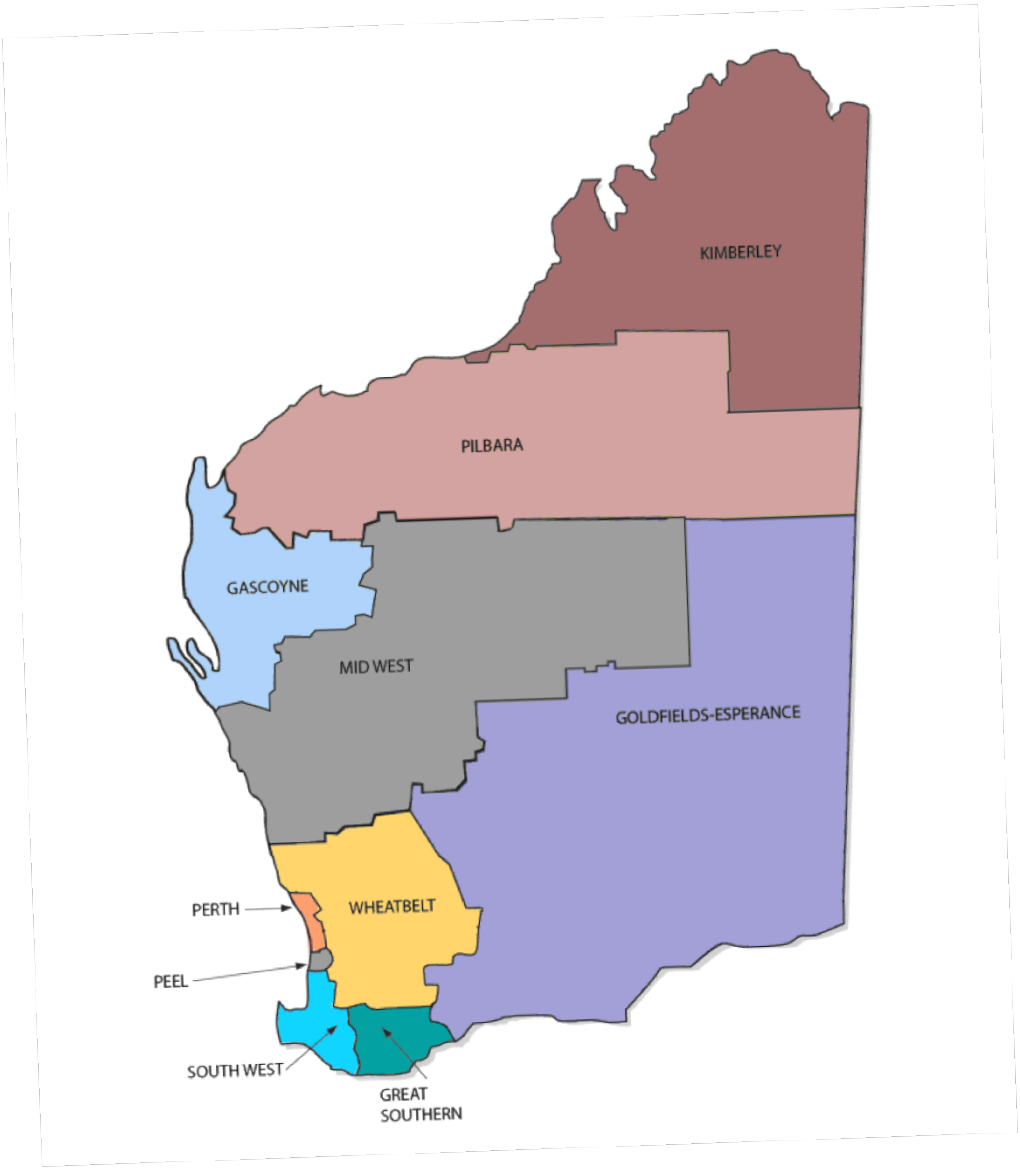
that had collected at the bottom of the specimen storage bag was harvested using a disposable non-sterile pasteur pipette (201C, Copan Diagnostic Inc.) and stored in 2 ml free standing, screw top, serum tubes at -20°C until required.

2.2.3. Regional Classification of Western Australia

Different government departments and organisations employ different regional classification systems for Western Australia. The Department of Local Government and Regional Development divides WA into ten development regions including Perth, Peel, Great Southern, the Southwest, Goldfields-Esperance, the Mid West, the Wheatbelt, Gascoyne, the Pilbara and the Kimberley (*Figure 2.2*) (Department of Local Government and Regional Development 2007). It is important to differentiate these regions from the *Management Regions* (*Figure 2.5; Section 2.3.9*) defined by the Department of Environment and Conservation for collation of kangaroo harvest figures (Department of Environment and Conservation 2008). Both systems of classification are used in this thesis.

Figure 2.2 Regional Zones of Western Australia defined by the Department of Local Government and Regional Development

(Department of Local Government and Regional Development 2007)



2.2.4. Analysis of Kangaroo Harvest Data

The average harvest yield in each *Management Region* between 1990 and 2006 and the number of kangaroos harvested per 100 km^2 for each DEC *Management Block* were calculated using data from the monthly returns logs submitted to DEC by professional shooters. Maps were then prepared to display the data using IDRISI Andes software (*Figure 2.5* and *Figure 2.6,* respectively). In preparing *Figure 2.5*, the total number of kangaroos harvested in each *Management Region* over the data period were averaged and classified according to the following; >10,000, 1,000 – 10,000 or <1,000 kangaroo per year. In preparing *Figure 2.6*, the size of each *Management Block* was determined using the area function available in IDRISI Andes and then used to calculate the number of kangaroos harvested per 100 km^2 *(Figure 2.6*) (Clark Labs 2007).

The cost of sample collection and transport by a dedicated investigator and a professional shooter were estimated (*Figure 2.2*) using official travel rates published by Murdoch University (Murdoch University 2009). These costs will vary depending on where harvesting takes place. The salary cost of employing a dedicated investigator was calculated based on the rate paid by Murdoch University to a graduate research assistant (HEW Level 5.5) (www.research.murdoch.edu.au/grants/salaries.html). The total time spent travelling and collecting samples from Capel during this study was used to estimate the number of hours required to undertake a similar investigation by an independent investigator. No allowance was made for payment of overtime or late night penalties. When employing a professional shooter to assist in sample collection, the cost of transport

from Capel to Murdoch was the amount charged to Murdoch University by a commercial courier company.

2.3. Results

2.3.1. Recruitment of Professional Shooters

A total of 17/40 professional shooters in selected geographic regions responded to the questionnaire. Eight individuals volunteered their assistance, two requested further information and seven declined to participate. Those unwilling to assist cited 'unable to shoot regularly enough to meet project requirements' as the primary reason for not being involved. No shooter ticked the 'project not of interest' box. Two shooters who volunteered their assistance, were recommended by King River International and the Arboviral Surveillance and Research Laboratory (ASRL). An additional two shooters were sourced by word of mouth using recommendations from shooters participating in the study. There was no association between the length of time the shooter had been working in the industry and their willingness to participate in the project. However, those shooters who had been involved in the industry the longest remained associated with the study until its completion. A number of shooters who were unable to assist in sample collection expressed an interest in the project and supported the study.

Professional shooters were initially involved in the project on a purely voluntary basis. Within two to three months of distributing sample collection kits, all but four shooters had ceased participation in the project (shooters A-D). To ensure continued participation, a financial incentive was provided. A total of $4.00 per blood sample was paid to individuals willing to collect blood themselves and $2.00 per sample was paid if the shooter was accompanied by the investigator. When the shooters began

collecting faecal samples in addition to serum, the fee was increased to $8.00 and $4.00 respectively. All four shooters (A – D) remained with the project following the introduction of this incentive.

2.3.2. Professional Shooter Profiles

Professional Shooter A is a resident of Capel. He has worked as a full time farmer and part-time kangaroo shooter for twenty five years. He had contracts with two commercial pet food processors during the course of this project and harvested kangaroos for approximately 7 – 10 nights each month, following the full moon. Professional Shooter A had previously participated in a research project undertaken by the Arboviral Surveillance and Research Laboratory (ASRL) and Mosquito-borne Disease Control Branch (MBDC) of the WA Department of Health. He participated in collecting samples between June 2006 and March 2009 for this study. For the first twenty four months, the investigator accompanied Shooter A on one occasion each month to collect samples. During this time, a number of alternative methods of specimen collection were evaluated to determine the most efficient means of gathering blood, tissue and faecal material at the field level. These techniques were then conveyed to Professional Shooters B and C who volunteered to collect samples for the research without assistance. For the remainder of his involvement, Shooter A collected specimens alone. Where possible, blood samples were collected by cardiac puncture, with the occasional sample obtained by free-catch from the neck or tail if blood could not be taken from the heart. Professional Shooter A sampled an average of 26 (23 – 29) adult and subadult kangaroos a night.

Professional Shooter B is a resident of Manjimup and the President of a local Professional Shooters Association. He harvested kangaroos for 7 – 10 nights per month, following the full moon. Samples were collected from Manjimup and a number of surrounding localities in the southwest of WA including; Balingup, Boyup Brook, Bridgetown, Greenbushes, Nannup, Northcliffe and Scott River. Professional Shooter B collected paired blood and faecal samples on a monthly basis throughout the study with no additional assistance from the investigator. He was also involved in developing a simple tagging system that enabled carcasses sampled in the field to be identified at the processing plant. Therefore, tissue samples taken from kangaroos during processing could be matched with blood and faecal samples collected immediately after harvesting. Professional Shooter B was the only individual involved in the research to harvest kangaroos for human consumption. He harvested an average of 26 (23 – 29) animals per night during the 30 months he was enrolled in the study.

Professional Shooter C is a resident of Perth and a retired meat industry worker with experience in carcass inspection. He travelled to properties in Eneabba, Badgingarra and Preston Beach to harvest kangaroos for pet meat. He harvested kangaroos throughout the year with less emphasis placed on the full moon. Professional Shooter C collected paired blood and faecal samples for approximately eighteen months of this study without any additional assistance from the investigator. Professional Shooter C sampled an average of 26 (21 – 32) animals per night during the study.

Professional Shooter D was a resident of Perth who ran a retail outlet for firearms and ammunition. He had been involved with a number of other research projects requiring kangaroo sample collection and offered extensive support to this project in its initial

stages. Professional Shooter D was contracted by DEC to manage a cull of kangaroos at Thomsons Lake Reserve during 2006 – 2007. During this period, the investigator accompanied Shooter D and his colleagues during harvesting to collect blood samples. After the Thomsons Lake cull was complete, Professional Shooter D no longer assisted in this research as his shoots around Perth were irregular and yielded too few animals.

2.3.3. Sample Collection

A total of 2603 blood, 260 faecal and 256 diaphragm muscle samples were collected from harvested western grey kangaroos (WGKs) (*Macropus fuliginosus*) throughout Western Australia by Professional Shooters A, B, C and D, between May 2006 and March 2009. The breakdown of the age and sex of each kangaroo harvested is provided in *Table 2.1*. Sample collection was completed predominantly in the field within 5 to 40 minutes of the animal being shot. A number of diaphragm muscle samples were successfully collected from animals at the processing plant. Undertaking sample collection from animals stored in the field chiller was considered impractical.

Blood collection by cardiac puncture was possible up to 40 minutes after each kangaroo had been shot. It was cleaner than collection by free-catch and interfered least with the shooter's harvesting routine. Blood collection by free-catch was most easily collected from the jugular vein and carotid artery if collected immediately after the animal was shot. Collection was easiest if the neck and vessels were severed but the head remained attached. This enabled the collector to grasp an ear to pull back the head during collection to direct the flow of blood away from the fur of the neck and

leaking oesophagus, minimising contamination. It was often difficult to collect large volumes even shortly after death if rapid and extensive bleeding had occurred as a result of the bullet wound. If insufficient blood was obtained, it was possible to increase the volume slightly by having a second person pump the rib cage to force blood from the heart during collection.

2.3.4. Transportation of Samples

When shooters collected blood and faecal specimens without assistance from the investigator, a commercial courier company was employed to deliver the samples to the laboratory within 24 hours of collection (ie. using an "overnight" courier). Significant delays in transportation occurred when samples were collected on a Friday or Saturday night because the courier was not able to pick them up until the following Monday, for delivery on the Tuesday. On two occasions, sample delivery was delayed by up to five days. Despite this delay, there was no evidence of haemolysis in the blood samples.

Table 2.1 The number of kangaroos sampled in each age and sex category by Professional Shooters A, B, C and D from fifteen collection locations in Western Australia

	Shooter A			Shooter B			Shooter C			Shooter D			Total
Category	**n**	**%**	**95% CI**	**n**	**%**	**95% CI**	**n**	**%**	**95% CI**	**n**	**%**	**95% CI**	
Sex													
Male	478	57.4	54.0, 60.7	458	59.5	56.1, 63.0	356	52.7	49.0, 56.5	179	54.9	59.5, 60.2	**1471**
Female	331	39.8	36.5, 43.1	310	40.3	36.9, 43.8	319	47.3	43.5, 51.0	138	42.4	37.1, 47.8	**1098**
Unknown	24	3.0	1.9, 4.3	1	0.3	0, 0.81	0	0	0	9	3.1	1.4, 5.2	**34**
Age													
Adult	676	81.1	78.4, 83.7	724	94.0	92.3, 95.6	370	54.8	51.0, 58.5	188	57.6	52.3, 62.9	**1958**
Subadult	54	6.6	5.0, 8.4	44	5.8	4.3, 7.6	39	5.9	4.2, 7.8	116	35.7	30.6, 40.9	**253**
Pouch Young	98	11.9	9.7, 14.1	0	0	0	0	0	0	3	1.2	0.2, 2.8	**101**
Unknown	5	0.7	0.2, 1.4	1	0	0, 0.8	266	39.4	35.8, 43.1	19	6.1	3.7, 9.0	**291**
Total	**833**			**769**			**675**			**326**			**2603**

2.3.5. Selection Bias

2.3.5.1. Comparison of the Number of Male and Female Kangaroos Harvested

Significantly more male kangaroos (57.3%; 55.3 – 59.2) were harvested compared to female kangaroos (42.7%; 40.8, 44.7) by Professional Shooters A, B, C and D combined, throughout the duration of the study *(*$p<0.05$) (*Table 2.1*). However, Professional Shooter C harvested a significantly lower proportion of male kangaroos compared to Professional Shooter A ($p<0.025$) and Professional Shooter B ($p<0.01$).

Between 1997 and 2006, a similar trend was also noted throughout the combined harvesting industry in Western Australia where a greater number of male kangaroos, both western grey (*Figure 2.3*) and red (*Figure 2.4*), were harvested than female. Despite this trend, the percentage of harvested western grey and red kangaroos that were male decreased significantly from 63.7% (63.4, 64.0) and 60.3% (60.1, 60.6) respectively, in 2003, to 54.8% (54.6, 55.1) and 48.3% (48.0, 48.6) respectively, in 2006 ($p<0.001$).

Figure 2.3 Proportion of male to female, western grey kangaroos (*Macropus fuliginosus*) harvested by all professional shooters in Western Australia (1997 – 2006)

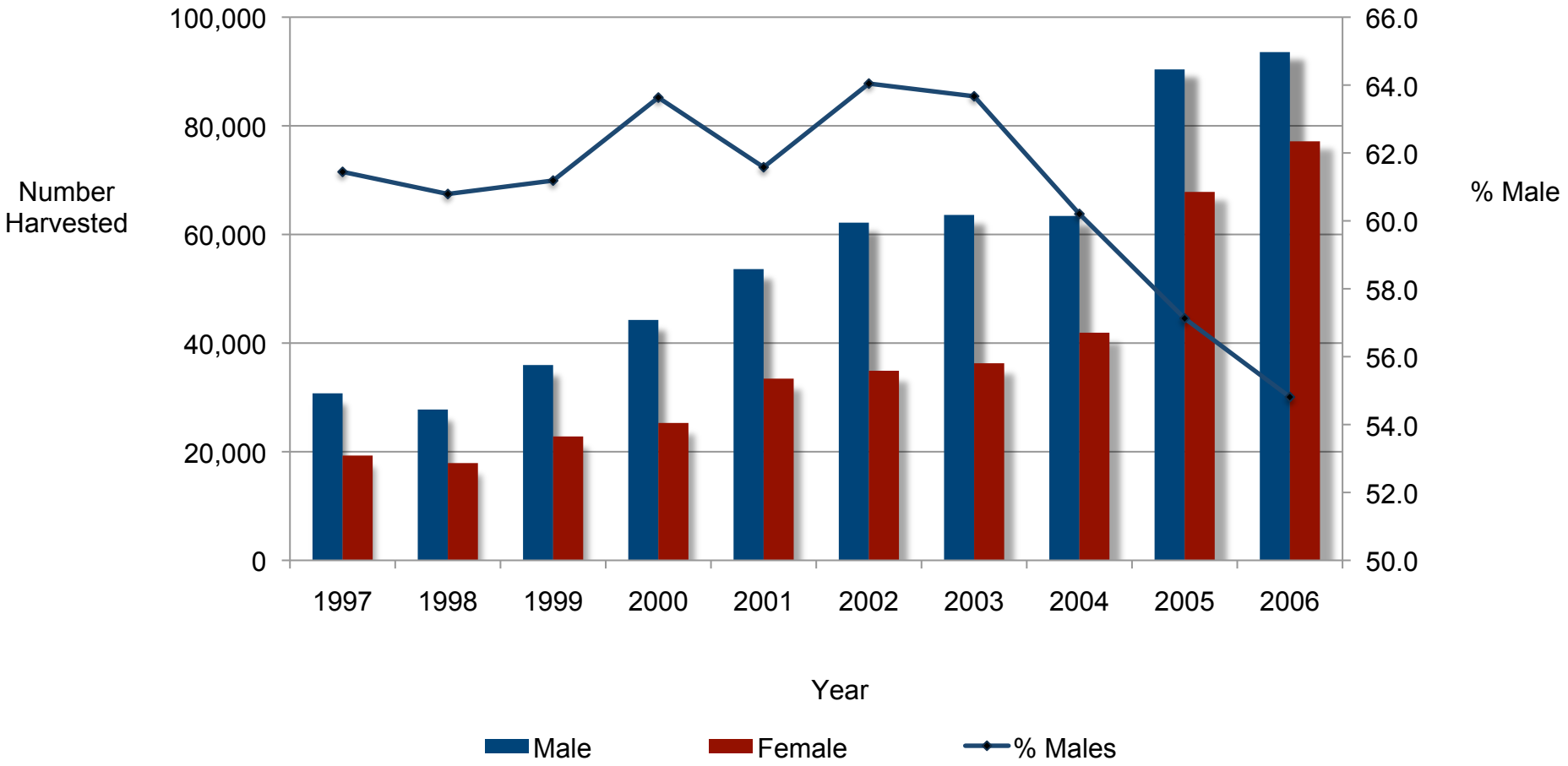
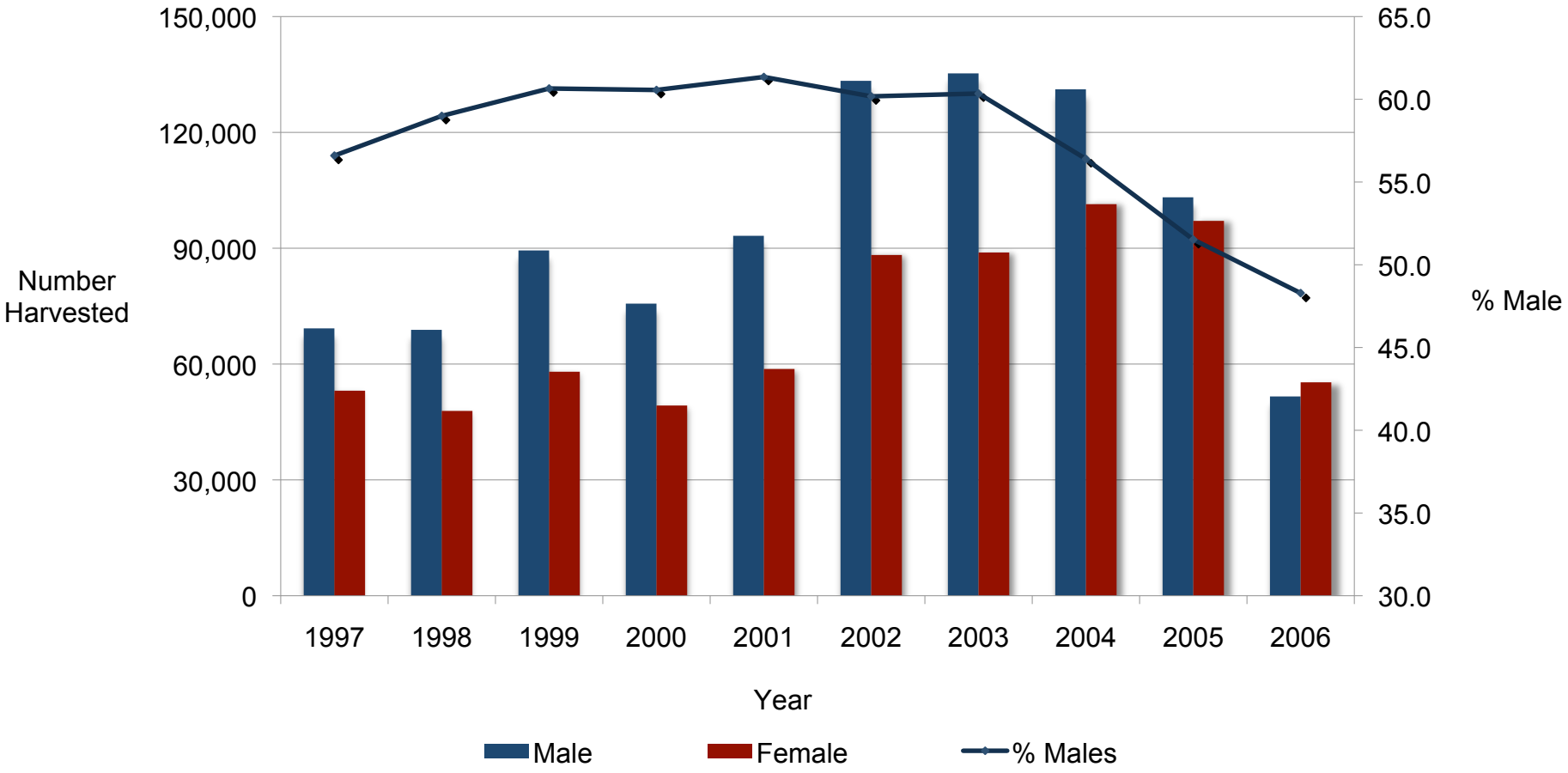


Figure 2.4 Proportion of male and female, red kangaroos (*Macropus rufus*) harvested by all professional shooters in Western Australia (1997 – 2006)



2.3.5.2. Comparison of the Number of Adult and Subadult Kangaroos Harvested

Significantly more adult kangaroos were harvested than subadult kangaroos during this study by Professional Shooter A, B, C and D ($p<0.05$). Whilst only 5 – 7% of the combined harvest yield from all four individuals were classified as subadult kangaroos, Professional Shooter D harvested significantly more subadult kangaroos compared to Professional Shooters A, B and C, representing 35.7% of his harvest yield ($p<0.001$).

2.3.6. Comparison of the Cost of Cardiac Puncture and the Free-Catch Technique for Blood Collection

A comparison of the estimated cost of obtaining a blood sample using each method of collection is presented in *Table 2.2*. The cost of collecting 100 samples using the free-catch and cardiac puncture methods was $42.00 and $78.80 per 100 blood samples, respectively. The additional costs for cardiac puncture included the purchase of a vacutainer holder for the needle and a 1.4 L sharps container for needle disposal that had a capacity of approximately 100 needles. Vacutainer holders were replaced after approximately 100-200 samples had been collected.

Table 2.2 Cost of the free catch and cardiac puncture methods of blood collection from kangaroos post mortem

Method	Size	Manufacturer	Cost Per Unit
Free Catch			
Plain Serum Tube	10 ml	SARSTEDT	$0.25
Disposable Pasteur Pipettes	5 ml	Copan Diagnostic Inc	$0.03
Serum Screw Tube	2 ml	Scientific Specialists Inc.	$0.05
Screw Tube Cap		Scientific Specialists Inc.	$0.09
TOTAL Cost/Sample			**$0.42**
Cardiac Puncture			
Vacuette Serum Tube	9 ml	Greiner Bio-One	$0.36
Vacutainer needle	18G, 1 ½"	Becton Dickinson	$0.21
**BD Vacutainer® Holder*		Becton Dickinson	$0.16
**BD Sharps Collector*	1.4 L	Becton Dickinson	$4.64
Disposable Pasteur Pipettes	5 ml	Copan Diagnostic Inc	$0.03
Serum Screw Tube	2 ml	Scientific Specialists Inc.	$0.05
Standard Screw Tube Cap		Scientific Specialists Inc.	$0.09
TOTAL Cost/Sample *(incl. non-regular purchases*)*			**$5.54**
TOTAL Cost/Sample *(excl. non-regular purchases*)*			**$0.74**

* *Non-regular purchases*

2.3.7. Comparison of the Cost of Blood Collection Using an Investigator or a Professional Shooter

A comparison of the cost of blood sample collection by a professional shooter and a dedicated investigator from Capel is presented in *Table 2.3*. The total cost of a dedicated investigator collecting 100 blood samples was $743.00 and the cost of employing a professional shooter to collect 100 blood samples was $408.00.

Table 2.3 Cost of collection of blood samples by a dedicated investigator and a professional shooter

Method	Cost Per Unit	No. Units	Cost Per 100 Samples
Investigator			
Motor Vehicle Allowance Rate (per kilometre)	$0.61	440	$268.40
Investigator (per hour)	$23.00	14	$322.00
Accommodation Expenses	$133.35	1	$133.35
Meal Allowance (Dinner)	$11.50	1	$11.50
Meal Allowance (Breakfast)	$7.75	1	$7.75
TOTAL (Cost/100 Samples)			**$743.00**
Professional Shooter			
Sample collection	$4.00	100	$400.00
Courier Australia fee	$8.00	1	$8.00
TOTAL (Cost/100 Samples)			**$408.00**

2.3.8. Meat Juice Sample Extraction

A total of 169 “one day old” diaphragm muscle samples were collected from kangaroos in Capel. The majority of samples produced in excess of 2 ml of meat juice, which was dark to bright red in colour with no pungent odour. Attempts to extract meat juice from 64 samples taken from carcasses at the processor were unsuccessful. The diaphragm samples were dry and tacky to touch and only small quantities of meat juice could be obtained. The meat juice sample was brown in colour with a pungent, acrid odour.

To determine whether carcass storage methods between harvesting and processing were responsible for the above change in specimen quality, 76 “one day old” diaphragm samples were divided into two equal-sized portions at the point of collection and stored at 4°C for 10 days prior to the freeze/thaw process. The volume of meat juice extracted was not as low as those sampled from the processor, but the fluid was similarly brown in colour with a pungent odour.

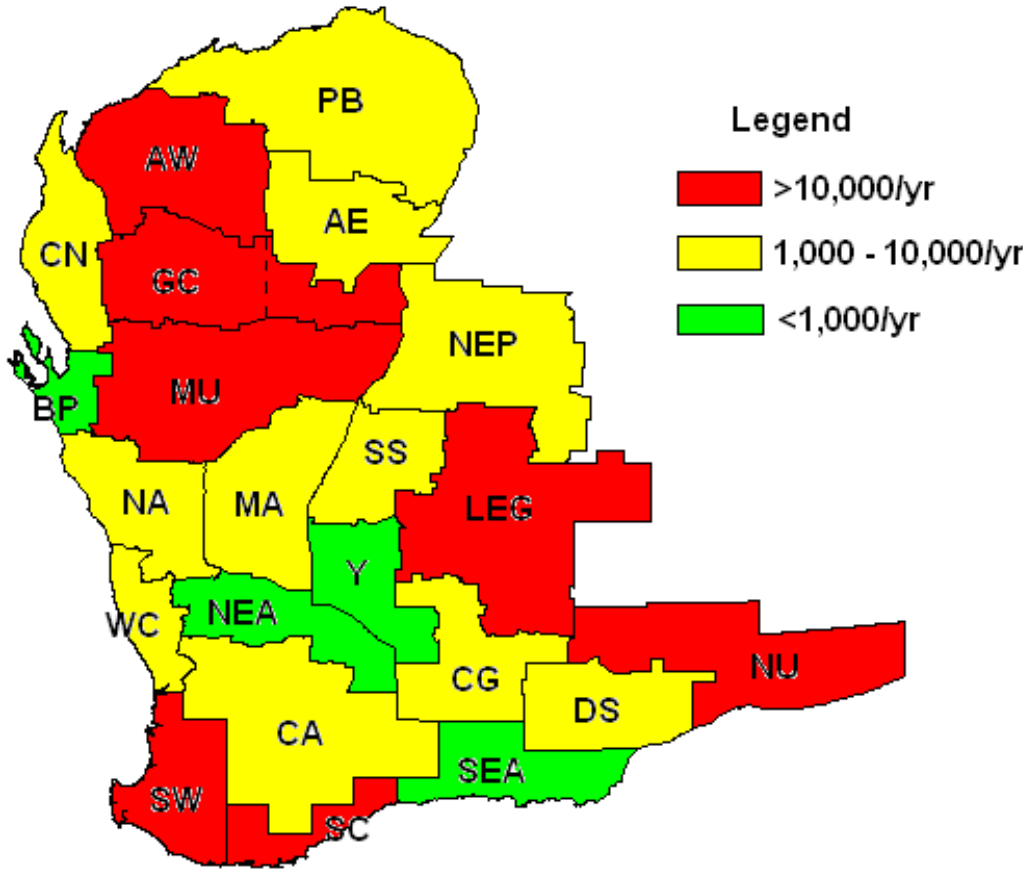
2.3.9. Analysis of Department of Environment and Conservation Harvest Data Pertaining to Disease Surveillance using a Geographical Information System

The number of kangaroos harvested in each of the individual *Management Regions* between 1990 and 2006 is displayed in *Figure 2.5*. Greater than 10,000 kangaroos were harvested from the Southwest, South Coastal, Nullabor, Leonora-Eastern Goldfields, Murchison, Gascoyne and Ashburton West *Management Regions* every year. Less than 1,000 animals were harvested annually from Bay Pastoral, North Eastern Agricultural, Yilgarn and South Eastern Agricultural *Management Regions.*

Between 1,000 and 10,000 animals per year were harvested from the all other *Management Regions.*

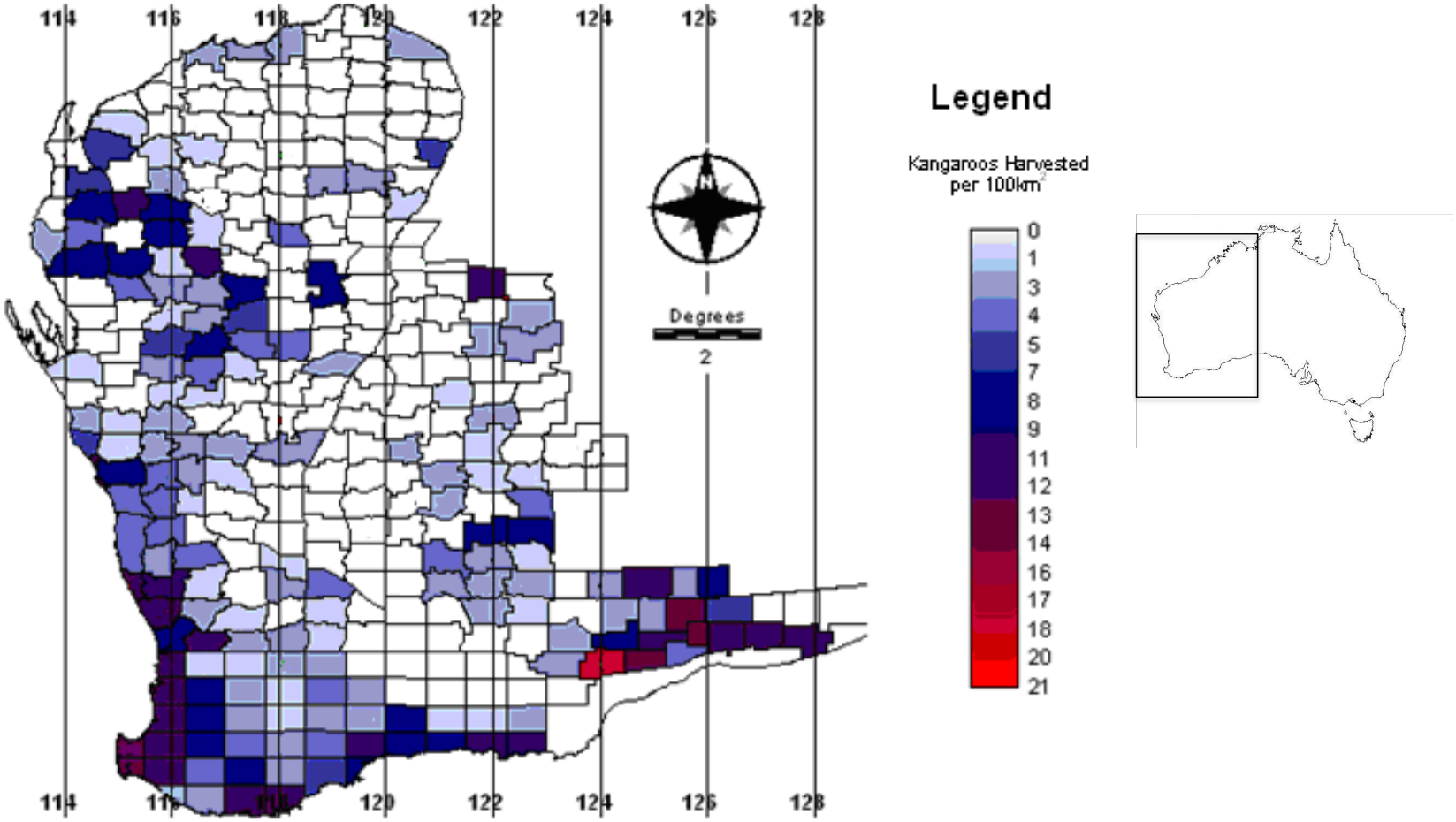
Kangaroo harvesting in Western Australia in 2006 was highly clustered, as evidenced by the three broad regions in WA where the intensity of harvesting was highest per 100 km^2 (*Figure 2.6*). These regions included the southwest of WA extending from Esperance to Geraldton, the northwest of WA inland from Carnarvon and an area east of Kalgoorlie, extending along the Nullabor. The greatest number of kangaroos were harvested from the second most southern *Management Block* in the region east of Kalgoorlie, intersected by the 124º line of longitude. Harvesting did not occur in a substantial number of *Management Blocks* throughout Western Australia.

Figure 2.5 The average harvest figures per management region in Western Australia (1990 – 2006)



Zone	Abbreviation	Management Region
Northern	AE	Ashburton East
	AW	Ashburton West
	PB	Pilbara
	CN	Carnarvon
	GC	Gascoyne (west)
Central	MU	Murchison
	NEP	North-East Pastoral
	GC	Gascoyne (east)
	MA	Magnet
	NA	Northern Agricultural
	BP	Bay Pastoral
	WC	Western Coastal
	SS	Sandstone
	Y	Yilgarn
South Eastern	LEG	Leonora-Eastern Goldfields
	CG	Coolgardie
	DS	Dundas
	NU	Nullarbor
	NEA	North Eastern Agricultural
	SEA	South Eastern Agricultural
	CA	Central Agricultural
	SC	South Coastal
South Western	SW	South Western

Figure 2.6 The density of kangaroos harvested in each management block in Western Australia (2006)



2.4. Discussion

2.4.1. Practicality of Utilising the Kangaroo Harvesting Industry for Disease Surveillance

The collection of blood, faecal and diaphragm muscle samples from harvested kangaroos was simple and efficient when undertaken in the field by either the shooter or by a dedicated investigator. The samples collected were of a high quality and generally free from contamination because of the short duration between sample collection and processing and the sterile collection techniques adopted. Field sampling also provided the only means of obtaining a whole blood sample from kangaroos, which is a required for many diagnostic assays.

There are few restrictions on the range of samples available for collection when sampling from kangaroos in the field. However, consideration has to be given to which retail market the shooter is supplying, as differences in evisceration protocol exist. If the animal is destined for human consumption, shooters are not permitted to open the thoracic cavity because this must be intact and available for inspection during processing. Consequently, samples of the heart, lung and diaphragm samples cannot be collected from these kangaroos in the field. It is also inappropriate to damage muscle groups that will later be processed into whole meat cuts as this reduces the profitability of the carcass for the shooter. It is also likely to be difficult to collect intact brain and ocular samples because animals are killed by a single shot, which causes extensive damage to the head and associated organs.

A major disadvantage of using a dedicated investigator to collect samples from kangaroos in the field is the cost associated with exercise. In this study, it was

estimated to cost $335 more for the investigator to collect 100 blood samples from kangaroos at Capel, compared to employing only a professional shooter at $4.00 per sample. This cost difference is likely to increase as the distance travelled by an investigator to reach remote collection sites also increases. The legislative requirement for carcasses to be refrigerated at a temperature less than 7°C, both during field storage and transport, provides a useful means of maintaining specimen quality during storage, prior to courier transport, when shooters are responsible for sample collection.

2.4.2. Recruiting and Maintaining the Assistance of Professional Shooters for Surveillance

The identification of professional shooters for this study was simplified because DEC maintains a record of the contact details of all individuals licensed to harvest kangaroos in Western Australia (*Wildlife Conservation Regulation 6 Licence)*. The initial selection of shooters, according to the geographic location in which they resided, was made to increase the geographic spread of samples and to ensure that courier transport would be feasible if required. Whilst this was a very efficient method of selection, the residential address registered with DEC did not always match the region in which the shooter harvested kangaroos. It would have been more useful to conduct a search of the registered properties in which individuals shot rather than their residential postcodes. Shooters could also be approached to assist in surveillance efforts based on the number of kangaroos harvested annually, to enable a large sampling frame to be used. This is a potentially more efficient method of shooter selection, as a number of individuals holding a *Wildlife Conservation Regulation 6 Licence* do not actively shoot kangaroos for various reasons, reducing the potential

pool of recruits. The observation that a small number of shooters collected the majority of samples is important because it means that fewer individuals will be required to maximise sample sizes. It will however limit the geographic spread from where samples are collected. If employing shooters who yield fewer animals each year, data will need to be aggregated over a period of time.

The *Australian Game Meat Hygiene and Handling* course is designed to educate professional shooters on the importance of maintaining adequate levels of hygiene during harvesting for food safety purposes (Technical and Further Education Commission NSW 1999), however little information is available on the risk of zoonotic disease transmission for this occupational group. Consequently, a large number of professional shooters actively expressed their support for this investigation into infectious diseases in kangaroos, despite being unable to meet the requirements to assist with sampling. Given this positive attitude amongst industry workers, it may have been possible to recruit and retain a larger cohort of shooters had a financial incentive been offered from the project's inception and less stringent sampling guidelines been imposed. Whilst not all licence holders actively harvest kangaroos, it is important to note that all professional shooters have passed a firearms competency test and have completed the *Australian Game Meat Hygiene and Handling* course (Pople and Grigg 1999; Department of Environment and Conservation 2008). They therefore form a valuable group of skilled individuals that may be able to assist in larger scale disease surveillance programmes if required.

2.4.3. Comparison of Blood Collection Techniques

Cardiac puncture was the preferred method of blood collection in this study because it provided the most sterile sample and reduced the likelihood of microbial contamination. A small amount of training was required to demonstrate how to use this technique safely to shooters. It was however, quicker, cleaner and more efficient than using the free-catch method of blood collection. This method also interfered least with the shooter's normal harvesting routine as it was possible to collect a sample up to forty minutes after an animal had been shot, either on the ground or after it was hung on the truck. The major disadvantage of this method was the requirement for additional equipment, which increased the cost per sample. With a fast-paced field situation and poor lighting, needle stick injuries may also occur. Shooter A had previously collected blood using the free catch method but quickly adopted the cardiac puncture technique after comparing the two. He felt it was both quicker and cleaner and was his preferred method of collection (Professional Shooter A, Personal Communication, 12th September, 2006).

A significant advantage of utilising the free-catch technique was its simplicity. This technique enabled large numbers of blood samples to be collected in the field with limited or no requirement for training and no additional equipment other than a blood tube. It was a less hygienic method of sample collection and became difficult to avoid contamination of the collector's hands and clothes with blood. A major disadvantage of this method was the difficulty in obtaining sufficient blood if there was more than a five minute delay between the fatal shot and sample collection. Blood could be expelled from the heart by pumping the rib cage to improve the efficiency of collection. However, this action did tend to increase the risk of contamination of the

sample because the oesophagus was often severed at the same time as the jugular and carotid vessels and stomach contents were often expelled with the blood. Both Professional Shooters B and C used this method of collection over the course of this research. Interestingly, Shooter B did not experience problems with contamination and Shooter C did. Less contamination was also achieved if the neck was severed but the head remained in place, enabling the collector to grasp an ear and direct the stream of blood away from the animal's fur and leaking oesophagus. Visualisation of the blood stream using a head torch also assisted in minimising contamination.

Cardiac puncture costs, on average, $36.80 per 100 samples more compared to the free-catch method. Had the cardiac puncture technique been used to collect all 2603 samples in this study, this would have amounted to an additional $950, compared to the cost of using the free-catch technique alone. Given the advantages of blood collection by cardiac puncture, this additional cost is justified because it would provide greater overall reliability and a better quality of samples. Furthermore, the savings associated with employing a professional shooter to collect samples would offset the costs associated with using the more expensive blood collection technique.

2.4.4. Sampling Kangaroo Carcasses at the Processor and Chiller

Sampling kangaroo carcasses at the processor was simpler than sampling in the field, however, there were a number of practical limitations. The choice of specimens available for collection at this stage were limited because the animals had been eviscerated in the field and were dead for up to ten days prior to processing. Most significantly, blood collection was not possible. Storage of carcasses in the field chiller prior to transport, and in the holding room prior to processing, also led to

surface bacterial contamination of tissue specimens, reducing their diagnostic quality. Given the sampling interest is available, however, the processor does provide a central location where large numbers of kangaroos from a wide range of geographic locations can be sampled. As the thoracic cavity remains largely intact for inspection in kangaroos intended for human consumption, the processing plant can provide a good opportunity to collect lung and heart samples.

Further research is required to investigate alternative methods to acquire serum samples direct from the processor for use in common diagnostic tests. The use of diaphragm derived meat juice in Denmark has been highly successful in the screening of *Salmonella* in pigs at the slaughter house (Nielsen, Ekeroth et al. 1998; Czerny, Osterkorn et al. 2001; Hannover 2003). This method is now being extended for use in surveillance of Ausjeszky's disease (De Lange, Haddad et al. 2003) and *Trichinella* (Beck, Gaspar et al. 2005). Diaphragm muscle from kangaroos is available for collection both in the field and at the processor and is considered a waste product. Sample collection is simple, but contamination and desiccation of muscle resulting from prolonged storage of carcasses prior to processing can limit its usefulness. Filtering of meat juice samples may assist in overcoming sample contamination of samples sourced from the processor.

Sampling at the field-based chiller facility was not practical because the chillers were small and crowded. When the chiller was full, it was not possible to reach all carcasses because they were stored in very close proximity to one another. Carcasses were added to the chiller after each night of harvesting making it difficult to predict how many carcasses would be available for sampling at any one point in time. In

addition, there was very little advantage in sampling from the chiller compared to the processor because the majority of evisceration was undertaken in the field. In kangaroos intended for human consumption, the heart and lungs were still available for sampling from the chiller but it was more cost effective and efficient to collect these samples at the processor.

2.4.5. Transportation of Samples

Transportation of specimens from the field to the laboratory where samples were collected by a professional shooter was simple given courier services ran regularly. Transportation times using commercial couriers were generally short, with delivery guaranteed within 48 hours. The absence of haemolysis on the two occasions when transport was delayed up to five days suggested that whole blood was able to withstand less than optimum conditions for this length of time with no significant deterioration in the diagnostic quality of specimens. The temperature at which the samples are exposed to during prolonged storage times will likely influence the amount of haemolysis that occurs (Gershfeld and Murayama 1987).

It may be possible to overcome additional costs associated with using a commercial courier by transporting samples from the field to a central-based processor in the carcass transport truck. Carcasses were transported from the field chiller to the processor using a refrigerated truck within five to seven days following the first night of harvesting. In 2006 there were 25 processing plants located throughout Western Australia, six of which were located in Perth. The remainder were located in major rural towns throughout the state, including Carnarvon, Jurien Bay, Esperance, Karratha, Augusta, Albany, Collie, Kalgoorlie, Geraldton and Leonora (Department

of Environment and Conservation 2006). Samples delivered to the processor by truck could be collected by an investigator at this point or further transported by commercial courier to the laboratory. This method of transport is likely to be most useful in remote Western Australia where a regular courier service does not run, fees are higher or transport times are long. Samples are less likely to deteriorate when stored and transported in a chilled environment. The disadvantage of this system is that samples are likely to endure long storage times prior to transport. Carcasses, and therefore samples, may remain in the chiller for seven to ten days before they are transported to the processor (Technical and Further Education Commission NSW 1999). Despite the chilled environment, delays of this magnitude may interfere with the quality of the specimens. To overcome this problem, shooters can be encouraged to collect samples over the one to two nights prior to the transport of carcasses from the chiller to the processor. This will minimise the delay between collection of samples and delivery to the laboratory.

2.4.6. Determining the Size of the Sample Population Available for Surveillance through the Kangaroo Harvesting Industry

The potential sampling population made available for surveillance using the harvesting industry is limited only by the commercial quota set for each calendar year. The annual quota for WGKs falls between 12-15% of the total estimated population and the quota for red kangaroos is approximately 20% of the total population (Pople and Grigg 1999; Department of Environment and Conservation 2008). Whilst the quota provides a reasonable forecast of the maximum source population for sampling, it is likely that a lesser number will actually be harvested in any given year. The number of red kangaroos harvested in relation to the annual quota is substantially

lower than the WGK (see *Table 1.2; Section 1.4.6*), due to the unpredictable nature of environmental conditions where reds predominate.

Analysis of historical harvest data provides a more accurate method of predicting the likely sample population that will be made available over the forthcoming year than the commercial quota. Over the past six years, the combined number of harvested red and WGKs has exceeded 200,000 animals annually (Department of Environment and Conservation 2008), providing a substantial sampling population for disease surveillance. It is reasonable to assume that the feasibility of sampling such a large number of free-ranging wildlife species through traditional methods of trapping, sedating and releasing, would be near impossible. The ethical issues associated with such an undertaking would also be numerous.

The importance of selecting an appropriate sample size in surveillance studies cannot be overemphasized as it ensures the results of a study are statistically meaningful (Webb, Bain et al. 2006). Whilst wildlife sample sizes are often selected on the basis of the availability of funding and convenience, the sample size should be derived from a systematic calculation wherever possible (Whitely and Ball 2002). Whilst maximising the number of animals in a study reduces random error, exceeding the ideal sample size can waste resources and present ethical issues (Altman 1980). The latter is a particular problem in wildlife studies that require animals to be trapped and sedated or alternatively sacrificed for the sole purpose of the investigation. A significant advantage of utilising the kangaroo industry for surveillance is that there are very few ethical issues relating to maximising the sample size because animals are not killed specifically for sampling purposes.

The provision of a kangaroo population estimate by DEC is also an invaluable dataset that is often lacking in studies of free-ranging populations. Many wildlife studies are limited by problems related to data acquisition and interpretation, missing or inaccurate denominator data and poor study design (Stallknecht 2007). Population estimates are useful in determining appropriate sample sizes necessary for disease detection, demonstration of freedom from disease and for the analysis of survey data.

2.4.7. Traceability within the Harvesting Industry

Mandatory tagging of kangaroo carcasses provides an established method of tracing any carcass and/or skin back to where the kangaroo was shot and to whom it was shot by. As demonstrated by the recent outbreak of Equine Influenza, trace back plays a vital role in controlling an EID (Hammond 2007). Any small delay can have potentially profound consequences on disease establishment and spread. Given the free-ranging nature of kangaroos, the ability the trace a carcass in this manner is likely to play a vital role in the control of a disease outbreak in these animals.

2.4.8. Difficulties Associated with Inconsistent Geographical Classification of Western Australia in Surveillance

Various government departments and organisations throughout the state employ different regional classification systems for Western Australia. The Department of Environment and Conservation collates kangaroo population and harvest statistics using a series of *Management Zones, Regions* and *Blocks* specific to the harvesting industry. It is difficult to undertake a combined epidemiological analysis using simple techniques to include vector, livestock, environmental and human case data when each dataset is calculated using a different regional classification system. This could

be overcome through the use of GIS, which assists in integrating and analysing large, multidimensional datasets (Pfeiffer and Hugh-Jones 2002). Geographical information systems has been commonly used in research associated with diseases for which wildlife represent a reservoir of infection. It is particularly useful when investigating patterns of disease spread between free-ranging populations, domestic animals and humans (Pfeiffer and Hugh-Jones 2002).

2.4.9. Selection Bias

Due to industry limitations, sex, species and geographic-based selection biases are unavoidable. Whilst the validity of any scientific study can be threatened by systematic error (Webb, Bain et al. 2006), it is important to consider the impact that each bias will have on the results and how this can be minimised. Under-reporting of disease may occur in a sample population consisting predominantly of adults if visual recognition of clinical signs is more common in younger animals. Conversely, an adult-biased sample population is likely to have a higher seroprevalence to endemic disease due to increased exposure to the infectious organism and the nature of antibody retention (Kerr, Whalley et al. 1981). Disease susceptibility may also differ between male and female kangaroos or between kangaroos from different geographical locations. In designing a surveillance study, the disease in question, the diagnostic assay and the influence of bias on the data generated will need to be taken into consideration.

2.4.9.1. Gender-Based Selection Bias

Gender-based selection bias was prominent amongst professional shooters involved in the project. There was a clear tendency for shooters to target male kangaroos because they are larger and more profitable. Additionally, leaving females in a mob allows sustainability of the population through ongoing reproduction. Data provided by the DEC showed that the total number of male kangaroos harvested exceeded the total number of females in all years except 2006, where there were slightly more red females harvested than males (Department of Environment and Conservation 2008). Interestingly, the ratio of harvested male to female kangaroos (both red and western grey) has declined each year since 2003. The trend suggests that either the number of male kangaroos has declined or the incentive to harvest them is no longer as strong as in previous years. It is likely that a combination of selective harvest pressure combined with increased mortality in older, male kangaroos during drought reduced male numbers. Increased mortality in male kangaroos during times of feed shortage has been attributed to a greater nutritional requirement, relative to females (Newsome 1977; Robertson 1986; Norbury, Coulson et al. 1988; Arnold, Grassia et al. 1991). Despite the male to female harvest ratio nearing 1:1, selection bias is still prominent when considering female kangaroos outnumber males 3:1 in the wild (Newsome 1977; Johnson and Bayliss 1981; Norbury, Coulson et al. 1988).

It is interesting to note that males comprised over half of the harvest yield from the cull at the Thomsons Lake Reserve. The Department of Environment and Conservation had instructed professional shooters to remove kangaroos regardless of age or sex due to gross overpopulation. Assuming the male to female ratio of harvested animals was representative of the overall population, the observation that

there were more males in the population compared to females disagrees with all other ecology studies (Newsome 1977; Johnson and Bayliss 1981; Norbury, Coulson et al. 1988). The enclosed nature of the reserve may have limited normal population dispersal and altered patterns of sexual segregation. Alternatively, the recent drought may have had an increased adverse effect on the health of males compared to females, which may have increased the likelihood that they would be shot.

The prominent gender-based selection bias of kangaroos harvested commercially may decrease the validity of results if disease susceptibility differs between male and female kangaroos. The prevalence of some infectious organisms does differ between macropod sexes, such as *Echinococcus granulosus* (Barnes, Mortona et al. 2007) and *Toxoplasma* (Parameswaran, O'Handley et al. 2009), whilst others such as Herpes virus do not (Kerr, Whalley et al. 1981). The impact of systematic error in any scientific study will largely depend on the disease in question, what is being detected and the nature of the selection bias itself (Webb, Bain et al. 2006). The validity of results can be maintained as long as any potential bias is identified and accounted for accordingly. Alternatively, it may be possible to reduce the level of bias by randomly sampling a representative number of male and female kangaroos to reflect the natural gender ratio. This is easiest if sampling occurs at the processing plant.

2.4.9.2. Age-Based Selection Bias

Age-based selection bias was evident in the kangaroos sampled during this study because the majority of professional shooters volunteering their assistance targeted adult kangaroos to maximise profit. Less than 10% of the kangaroos sampled by Professional Shooters A, B and C were classified as subadult. This is less than the

estimated proportion of subadults in wild populations, which may be as high as 36.2% of the population (Arnold, Grassia et al. 1991). These observations may have been inaccurate because of the subjective nature of the assessment used to determine the age of the animals and the possibility that intra-observer and inter-observer reliability was not strong. The most accurate means of estimating a kangaroos age is to consider both tooth eruption and measurements of body size and proportion (Dawson 2002). However, this is not practical when working with professional kangaroos shooters due to the fast paced nature of the harvesting routine.

Interestingly, the percentage of subadult kangaroos harvested at Thomsons Lake Reserve was greater than 38% of Professional Shooter D's total harvest yield. Whilst this was likely a reflection of the non-biased nature of the cull at Thomsons Lake, it is also possible that misallocation bias influenced the outcome of the analysis in this study. A number of female kangaroos were possibly classified as subadults due to their relative small size in comparison to males.

As age is not recorded on the monthly returns log completed by shooters to generate DEC's harvest data, it was not possible to undertake an analysis of age-based selection bias across the entire industry. Its presence, however, is unavoidable as shooters are paid on a per kilogram basis and processors do not accept carcasses weighing less than 15 kg (Frank Zambonetti, King River International, Personal Communication, 17th March, 2009). This encourages shooters to target larger animals, which inevitably represent older animals. Minimum weight restrictions are in place as the overall price processors receive for kangaroo hides is determined by the proportion of large versus medium skins and small skins have little commercial value

(Frank Zambonetii, King River International, Personal Communication, March 17th, 2009).

2.4.9.3. Species-Based Selection Bias

A disease monitoring programme centred on the kangaroo harvesting industry in Western Australia limits surveillance to red and WGKs. Sampling may be extended to the euro (*M. robustus*) in years when populations are deemed adequate and a commercial quota is set, but this will not be consistent (Department of Environment and Conservation 2008). Given this limitation, surveillance in the red and WGK will still provide valuable data because these two species are by far the most abundant macropods in the state (Pople and Grigg 1999) and commonly come into close contact with livestock species and people. In addition, common arboviral vectors *Aedes camptorhynchus* and *Culex annulirostris* feed on these marsupials (Johansen, Power et al. 2004). Although macropod species tend to harbour similar infectious agents, Speare et al. (1989) noted that diseases in smaller macropodids are rarely reported (Speare, Donovan et al. 1989). Surveillance is therefore likely to be most sensitive in larger bodied, red and WGKs, compared to smaller macropod relatives.

2.4.9.4. Geographic Selection Bias

The kangaroo harvesting industry is highly clustered within selected *Management Blocks* throughout Western Australia. Approximately 50% of kangaroos are harvested by only 10% of the most successful professional shooters (Department of Environment and Conservation 2008). The clustered nature of the industry relates partially to the lack of infrastructure and rough terrain in remote Western Australia, where harvesting is not economically viable. The wet season (summer months) in the

Pilbara and Gascoyne development regions reduces harvesting activity for extended periods of time due to inaccessibility associated with flood. The supply of kangaroos for surveillance in the Mid West, Wheatbelt and Goldfields-Esperance regions is also inconsistent because harvesting depends on local farming routines. Shooters will often be contracted to reduce kangaroo populations only when farms are in crop because this is the time that kangaroos are considered a particular a nuisance (Professional Shooter A, Personal Communication, October 11th, 2006). Consequently, the highly clustered nature of the harvesting industry coupled with the small number of shooters harvesting a disproportionate number of animals will significantly reduce the number of individuals that are available to collect samples for surveillance. The most consistent harvesting activity, both temporally and spatially, takes place in the Southwest, Peel, Perth and Great Southern development regions. This is likely due to regular rainfall, abundant populations of WGKs and year round farming practices. Obtaining regular samples from kangaroos across these latter regions for a disease monitoring programme is therefore feasible.

Kangaroo harvesting activity appears to be distributed in a similar fashion to human population density, primarily because shooters work in close proximity to their own homes. According to the Australian Bureau of Statistics (2008) almost 90% of Western Australia's population is located in the south-west corner of the state, encompassing the capital city of Perth and stretching along the southern coastline to beyond Albany and east to Lake Grace (Tindall 2008). Whilst it is important for surveillance to reach as many geographical regions as possible, the cost associated with sampling kangaroos from across Western Australia's 2,531,600 square kilometres is economically unviable and possibly unnecessary. Surveillance priorities

are likely to be highest in areas where domestic animal stocking rates are highest, where kangaroos are in close contact with both people and livestock and where animal and human movement from interstate and overseas occurs. As the majority of harvesting in Western Australia occurs in these regions, geographical bias introduced in sampling kangaroos through the commercial harvesting industry is less likely to interfere with surveillance priorities. Using hypothesis-driven surveillance, careful design of study regimens can take advantage of this geographical bias by ensuring a specific hypothesis is established and appropriate surveillance methods are chosen to test it (Hoye, Munster et al. 2010). Hypothesis-driven surveillance aims to overcome the difficulties associated with surveillance in wildlife and favours a compromise between ideal sampling based on probability and the constraints of sample collection, transport and analysis (Hoye, Munster et al. 2010).

3. PREVALENCE OF *SALMONELLA* INFECTION IN WILD KANGAROOS IN WESTERN AUSTRALIA

3.1. Introduction

Macropods were first suspected as being reservoirs of *Salmonella* in the 1960s when contamination of kangaroo meat in pet food preparations was found to be a risk factor for *Salmonella* infection in pets (Anderson, Crowder et al. 1964). Countries importing kangaroo meat from Australia for human consumption also reported *Salmonella* contamination rates as high as 44.9% (Suzuki, Kawanishi et al. 1967). Following a case of salmonellosis in an infant on Rottnest Island, it was discovered that 71% of quokkas (*Setonix brachyurus*), which are small macropods, were also infected (Iveson and Bradshaw 1973). Further investigation revealed that the infection rate in quokkas peaked as high as 70 – 100% in summer, when feed quality declined and digestive physiology was disrupted, and dropped as low as 0 – 30% in winter, when feed quality improved (Hart, Bradshaw et al. 1985). Thomas et al. (2001) isolated *Salmonella* spp. from faecal and tissues samples from eastern grey kangaroos (*Macropus giganteus*), western grey kangaroos (*Macropus fuliginosus*), red kangaroos (*Macropus rufus)* and common wallaroos (*Macropus robustus*) over a period of 20 years. From the 57 animals sampled, 62 isolates of *Salmonella* spp. were cultured, belonging to 24 different serotypes. The majority of infected animals were captive or pet macropods, which is likely to have been a contributing factor to the high rate of infection (Thomas, Forbes-Faulkner et al. 2001). As with many macropod infections, *Salmonella* is more likely to lead to clinical disease in animals housed away from their natural environment. Hand-reared joeys often experience stress related to sudden

withdrawal from their mothers, a change in nutrition and unaccustomed exposure to humans and other animals. Between 1981 and 1985, Speare and Thomas (1988) examined 65 live and 38 dead joeys. A total of 26.8% were found to be infected with *Salmonella* spp. whilst 21.7% were actively excreting the bacterium in faeces (Speare and Thomas 1988).

Although it appears that salmonellosis is predominantly a disease of animals in captivity, contamination of kangaroo meat for human and pet consumption suggests that infection is also likely to occur in free-ranging macropods (Samuel 1982). Contamination rates were reportedly much higher in early studies (Anderson, Crowder et al. 1964; Suzuki, Kawanishi et al. 1967; Bensink, Ekaputra et al. 1991) and are likely to have decreased in more recent years following improved hygiene practices within the kangaroo harvesting industry (Eglezos, Sofroni et al. 2007; Holds, Pointon et al. 2008).

There have been no systematic studies to determine the prevalence of *Salmonella* in free-ranging kangaroos. Published studies have been limited to sampling carcasses at the processor, orphaned joeys and captive macropods (Suzuki, Kawanishi et al. 1967; Speare and Thomas 1988; Bensink, Ekaputra et al. 1991; Thomas, Forbes-Faulkner et al. 2001; Holds, Pointon et al. 2008). Until such an investigation is undertaken, it is not possible to comment on whether kangaroos naturally harbour larger quantities of *Salmonella* than domestic livestock species.

3.1.1. Aims of the Study

The aim of this study was to determine the prevalence of naturally acquired *Salmonella* infection in wild kangaroos from a range of geographical locations. Investigation was also undertaken to determine whether an association exists between faecal prevalence *o*f *Salmonella* and the age and sex of kangaroos, the location and season (quarter) in which samples were collected and the accumulated rainfall that fell prior to specimen collection.

3.2. Materials and Methods

3.2.1. Animal Data

Faecal samples were collected from western grey kangaroos (WGKs) (*M. fuliginosus*) at ten locations throughout the mid to southwest of Western Australia including: Capel, Manjimup, Nannup, Northcliffe, Boyup Brook, Bridgetown, Preston Beach, Eneabba and Badgingarra. A single, opportunistic collection was conducted at Whiteman Park in July 2007. A map displaying the approximate location of each sample collection site has been provided previously in *Figure 2.1.* For each sample, the location and date of collection were recorded as well as the sex and age of the animal. Shooters subjectively categorised kangaroos into subadult and adult age groups based on size and apparent sexual maturity (*Section 2.2.2.2)*.

3.2.2. Faecal Sample Collection

Faecal samples were collected from harvested kangaroos according to the method described in *Section 2.2.2.4*. Briefly, the intestines were incised during evisceration and between one and five faecal balls were massaged directly into individual specimen storage bags and were uncontaminated by hand. A record of the animal's

sex and age group (adult or subadult) was recorded. Faecal samples were refrigerated at 4°C within 24 hours of collection and delivered to the WA Department of Agriculture for culture within one to three days.

3.2.3. Faecal Sample Culturing

Samples were cultured by the Animal Health Laboratory, Department of Agriculture and Food, Western Australia. Positive isolates were serotyped by Pathwest, Sir Charles Gairdner Hospital, Western Australia, and the results reported to the National Enteric Pathogen Surveillance Scheme (NEPSS), Melbourne University.

3.2.4. Environmental Data

Daily rainfall data was obtained from the Bureau of Meteorology (BOM) for weather stations located closest to the sample collection sites (*Table 3.1)*. Accumulated rainfall was calculated for the preceding 30 and 60 days at each site for each date of collection. These times periods were chosen because they are consistent with the lag in Normalised Difference Vegetation Index (NDVI) noted in numerous studies (Roderick 1994; Damizadeh, Saghafian et al. 2001; Chandrasekar, Sesha Sai et al. 2006). The NDVI is an index used to monitor vegetation growth derived from satellite data. Rainfall records were not available for Whiteman Park so samples from this location were excluded from all analyses that incorporated rainfall. Rainfall in the preceding 30 days (RainCat30) and 60 days (RainCat60) were grouped into 4 categories (<25mm, 25-49 mm, 50-99 mm and ≥100mm) and (<50mm, 50-99 mm, 100-199 mm and ≥200mm), respectively. Data were also aggregated based on the quarter of the year in which they were collected: Q1=Jan-Mar; Q2=Apr-Jun; Q3=Jul-Sep; Q4=Oct-Dec.

Table 3.1 Bureau of Meteorology (BOM) weather station from which rainfall data was obtained for each sample collection site

Sample Collection Site	BOM Weather Station Number
Preston Beach	9679
Northcliffe	9590
Nannup	9585
Manjimup	9573
Eneabba	8225
Badgingarra	9037
Capel North	9992
Bridgetown	9510
Boyup Brook	9504

3.2.5. Data Analysis

A generalised linear model which assumed a binomial distribution for *Salmonella* shedding was fitted to the data to determine whether there was any association with sex, age, quarter or rainfall category (McCullagh and Nelder 1989). A separate analysis of the data from Capel was also undertaken because it was the only location where a large number of samples were collected across all rainfall and quarter categories as well as sex and age groups. Samples with unknown sex or age were excluded from this analysis. The CHI-square test was used to determine whether any two proportions were significantly different from each other, unless one of the cell values was less than five, in which case Fisher's Exact test was used. The 95% confident intervals surrounding prevalence levels were calculated using the Adjusted Wald Method or Modified Wald Interval because it provides best coverage for the specified interval when sample sizes are small (Agresti and Coull 1998).

3.3. Results

3.3.1. Distribution of Samples Across Sex, Age, Rainfall and Quarter Categories for all Locations

A total of 645 faecal samples were collected from WGKs at ten sites throughout Western Australia, ranging from 24 to 202 samples at each site (*Table 3.2).* Faeces were generally of a firm, dry ball-like structure, with the exception of a number of collections between May and June in which they were brighter green in colour and softer in consistency. This was particularly true for samples from Badgingarra, Preston Beach, and Boyup Brook. Consistency and colour returned to normal by July. The sex of each kangaroo was determined and recorded for all but three samples from Eneabba and two from Badgingarra. The number of males and females were well distributed across all sample collection sites (*Table 3.2*). Professional Shooter C did not consistently estimate the age group to which the kangaroos belonged. Consequently 203 kangaroos from Eneabba, Badgingarra and Preston Beach had no age group recorded. Age was not well distributed across the two categories, with only 42 out of a total of 400 animals considered subadult (*Table 3.2)*. The spread of samples across each accumulated rainfall category (*Table 3.3)* and quarter (*Table 3.4*) were well distributed in Capel only.

Table 3.2 The age and sex of kangaroos sampled at each study location

Location	Sex			Age			Total
	Female	**Male**	**Unknown**	**Adult**	**Sub-adult**	**Unknown**	
Badgingarra	37	63	2	0	0	102	**102**
Boyup Brook	18	12	0	30	0	0	**30**
Bridgetown	15	12	0	27	0	0	**27**
Capel	78	124	0	181	21	0	**202**
Eneabba	43	44	3	12	4	74	**90**
Manjimup	17	31	0	46	2	0	**48**
Nannup	34	31	0	55	10	0	**65**
Northcliffe	5	19	0	24	0	0	**24**
Preston Beach	18	9	0	0	0	27	**27**
Whiteman Park	12	18	0	25	5	0	**30**
Total	**277**	**363**	**5**	**400**	**42**	**203**	**645**

Table 3.3 Number of samples collected in each accumulated rainfall category at each location

30 Day Accumulated Rain Category (RainCat30)	**< 25mm**	**25–49 mm**	**50–99mm**	**≥ 100mm**
Badgingarra	44	30	0	28
Boyup Brook	0	30	0	0
Bridgetown	0	0	0	27
Capel	45	18	45	94
Eneabba	62	0	28	0
Manjimup	0	0	32	16
Nannup	31	0	34	0
Northcliffe	0	24	0	0
Preston Beach	0	0	0	27
Whiteman Park	0	0	0	0

60 Day Accumulated Rain Category (RainCat60)	**< 50mm**	**50–99 mm**	**100–199 mm**	**≥ 200mm**
Badgingarra	49	25	0	28
Boyup Brook	0	0	30	0
Bridgetown	0	0	0	27
Capel	45	63	30	64
Eneabba	37	53	0	0
Manjimup	0	32	0	16
Nannup	31	0	34	0
Northcliffe	0	0	24	0
Preston Beach	0	0	0	27
Whiteman Park	0	0	0	0

Table 3.4 Number of samples collected in each quarter at each location

Location	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec
Badgingarra	0	55	28	19
Boyup Brook	0	30	0	0
Bridgetown	0	27	0	0
Capel	24	57	62	59
Eneabba	25	37	28	0
Manjimup	0	32	16	0
Nannup	31	34	0	0
Northcliffe	0	0	0	24
Preston Beach	0	27	0	0
Whiteman Park	0	0	30	0

3.3.2. *Salmonella* Serotypes Isolated from Kangaroos in Western Australia

Twenty three positive results were obtained from a total of 645 faecal samples cultured from WGKs in WA. From the positive isolates, seven different serotypes were identified (*Table 3.5*). *Salmonella enterica* serovar Muenchen (12/23) was the most commonly isolated serotype, followed by *S.* ser. Kiambu (6/23).

Table 3.5 *Salmonella* isolates cultured from kangaroo faecal samples

Isolates	Number	Location
S. ser. Muenchen	**12**	Badgingarra (9), Eneabba (1), Boyup Brook (1), Whiteman Park (1)
S. ser. Kiambu	**6**	Capel
S. ser. Rubislaw	**1**	Badgingarra
S. ser. Lindern	**1**	Eneabba
S. ser. Champaign	**1**	Eneabba
S. ser. Saintpaul	**1**	Preston Beach
S. ser. II 42:g,t:-	**1**	Preston Beach

3.3.3. Association Between Location and the Prevalence of Faecal *Salmonella* in Western Grey Kangaroos from all Sample Collection Locations

The overall prevalence of infection in 645 kangaroos across ten sample collection sites was 3.57% (2.3 – 5.3). The individual prevalence estimates for each location are displayed in *Table 3.6.* Badgingarra had the highest prevalence at 9.8% (5.3 – 17.5), which was significantly higher than Capel (p=0.026), Manjimup (p=0.031) and Nannup (p=0.007). There was no significant difference in prevalence between the remaining collection locations.

Table 3.6 Estimate of *Salmonella* prevalence for kangaroos from each sample collection location

Location	n	Prevalence (%)	95% CI
Nannup	65	0.0 [a]	0.0, 4.8
Manjimup	48	0.0 [a]	0.0, 6.4
Bridgetown	27	0.0 [ab]	0.0, 10.9
Northcliffe	24	0.0 [ab]	0.0, 12.1
Capel	202	3.0 [a]	1.2, 6.5
Boyup Brook	30	3.3 [ab]	0.0, 18.1
Eneabba	90	3.3 [ab]	0.7, 9.8
Whiteman Park	30	3.3 [ab]	0.0, 18.1
Preston Beach	27	7.4 [ab]	1.0, 24.5
Badgingarra	102	9.8 [b]	5.3, 17.5

[ab] *Different letters represent significant difference in prevalence levels between locations ($p<0.05$).*

3.3.4. Association Between Accumulated Rainfall, Quarter, Age and Sex and the Prevalence of Faecal *Salmonella* in Western Grey Kangaroos from all Sample Collection Locations

There was a significant association between accumulated rainfall in the preceding 30 days and the prevalence of *Salmonella* spp. isolation across all sample collection locations (p=0.014) (*Table 3.7*). The prevalence of *Salmonella* in samples collected in the <50 mm RainCat30 category was significantly higher than in samples collected in the <25 mm rainfall category (p<0.05). The effect of accumulated rainfall in the preceding 60 days was not considered significant (p=0.337). Quarter was significantly associated with the prevalence of *Salmonella* after removing the effect of RainCat60 (p=0.026), and was close to significance after removing the effect of RainCat30 (p=0.073). The level of shedding was significantly higher in the Apr-June quarter

than in either the Jul-Sep or Oct-Dec quarters ($p<0.05$). A statistical interaction was found to exist between RainCat60 and location ($p=0.022$). There was no association between age and sex and the prevalence of *Salmonella*.

Table 3.7 Association between quarter and accumulated rainfall categories and the prevalence of faecal *Salmonella* in kangaroos from all locations

Category	Number Positive	Prevalence (%)	95% CI
Season			
Jan-Mar	1	2.0 [a,b]	0, 6.1
Apr-Jun	17	5.9 [a]	3.5, 9.0
Jul-Sep	3	1.2 [b]	0.4, 5.5
Oct-Dec	2	1.8 [b]	0.1, 7.3
RainCat30			
<25mm	3	1.9 [a]	0, 3.9
<50mm	10	11.5 [b]	3.3, 19.7
<100mm	4	3.8 [a,b]	0, 8.5
>100mm	5	2.1 [a]	0, 4.3
RainCat60			
<50mm	9	4.7 [a]	1.0, 8.4
<100mm	7	5.9 [a]	1.4, 10.4
<200mm	3	9.6 [a]	0, 21.4
>200mm	3	1.0 [a]	0, 2.4

[ab] *Different letters indicate statistically significant differences ($p<0.05$).*

3.3.5. Association Between Accumulated Rainfall, Quarter, Age and Sex and the Prevalence of Faecal *Salmonella* in Western Grey Kangaroos from Capel

The estimated prevalence of *Salmonella* spp. infection in WGKs from Capel was 3.0% (n=202; 1.2 – 6.5). The individual estimates for each of the quarter and accumulated rainfall categories are listed in *Table 3*.9. A significant association was found between rainfall in the preceding 60 days and the prevalence of *Salmonella* spp. in faeces (p=0.023), with shedding significantly higher in the 50 – 99 mm (6.3%) and 100 – 199 mm (6.7%) categories than all others, which had zero samples containing *Salmonella* spp. The effect of quarter was also very close to significance (p=0.056). Infection was significantly higher in the Apr-Jun period (7.0%) than in either the Jan-Mar or Jul-Sep periods when there were no positive samples at Capel (p<0.05). No association was found between RainCat30 (p=0.125), age (p=0.289) or sex (p=0.779) and the prevalence of *Salmonella* spp. isolation from kangaroo faeces from Capel.

Table 3.8 Prevalence of *Salmonella* isolation at Capel for each quarter and accumulated rainfall category

Category	Number Positive	Prevalence (%)	95% CI
Quarter			
Jan-Mar	0	0 [a]	0, 7.4
Apr-Jun	4	7.0 [b]	3.5, 9.0
Jul-Sep	0	0 [a]	0.4, 5.5
Oct-Dec	2	3.4 [a,b]	0.1, 7.3
RainCat30			
<25mm	0	0 [a]	0
<50mm	2	11.1 [a]	0, 25.6
<100mm	2	4.4 [a]	0, 10.5
>100mm	2	2.1 [a]	0, 5.0
RainCat60			
<50mm	0	0.0 [a]	0
<100mm	4	6.3 [b]	0.2, 12.4
<200mm	2	6.7 [b]	0, 15.7
>200mm	0	0 [a]	0

3.4. Discussion

3.4.1. Validity of Results

Isolation of *Salmonella* spp. from kangaroo faeces in this study indicates that WGKs are infected by the organism in their natural habitat. Samples were collected directly from the intestinal tract, ensuring that faeces were collected in a sterile manner. This minimised the chance of obtaining false positive results. The low prevalence is possibly an underestimation of the true level of infection in kangaroos, reflecting the difficulties associated with detection of intermittent shedders and carrier animals (Futagawa-Saito, Hiratsuka et al. 2008). The phenomenon of intermittent shedding is described in other organisms residing in the gastrointestinal tract, including and *Campylobacter* spp. (Jones, Howard et al. 1999). Culturing of tissue samples in addition to faeces, in particular mesenteric lymph nodes, has been found to increase the likelihood of detection of *Salmonella* spp. by 2.4 times (Speare and Thomas 1988) Studies have also demonstrated that the chance of isolating the bacterium increases with the amount of faeces used for culture (Funk, Davies et al. 2000). The likelihood of isolating *Salmonella* spp. in this study may have been improved through sampling lymph nodes and collecting a minimum of five faecal balls from each animal for culture. However, it is not possible to predict the magnitude of the change and hence the return on the additional effort and expense required.

3.4.2. Significance of the Kangaroo as a Reservoir Host of *Salmonella* in Western Australia

The prevalence of *Salmonella* infection in kangaroos in this study was notably lower than in previous reports of infection in captive macropods and contamination of carcasses at the processor (Samuel 1982; Hart, Bradshaw et al. 1985; Speare and

Thomas 1988; Bensink, Ekaputra et al. 1991; Eglezos, Sofroni et al. 2007; Holds, Pointon et al. 2008). Most significantly, the observed prevalence was also markedly lower than reported levels of infection in quokkas (*S. brachyurus)* on Rottnest Island (Iveson and Bradshaw 1973). This latter finding can be attributed to the fact that quokkas live in a closed population on the island and are exposed to abnormally high levels of contact with humans. During the summer months when feed availability decreases and digestive physiology is disrupted, it is likely that quokkas scavenge through food scraps left by tourists, increasing the risk of infection with *Salmonella* (Samuel 1982).

Investigations focussing on *Salmonella* infection in kangaroos have been largely limited to surveys in pets, captive macropods and hand-reared joeys. One such study isolated *Salmonella* spp. from 51% of kangaroos in the study population (Samuel 1982). Similarly high results have been published elsewhere (Speare and Thomas 1988; Thomas, Forbes-Faulkner et al. 2001). Given the low level of faecal isolation from kangaroos in this study, it is likely that *Salmonella* infection is more common in captive and hand-reared macropods than those living in their natural habitat. This anomaly has been reported in a range of other macropod diseases, including lumpy jaw and coccidiosis (Speare, Donovan et al. 1989). Stressors associated with environmental and diet related change are likely responsible for the elevated levels of infection with *Salmonella* reported in captive animals (Samuel 1982).

The carriage of *Salmonella* spp. in the gastrointestinal tract influences the risk of carcass contamination, and in turn the risk of human salmonellosis from consumption of kangaroo meat. In 1965 and 1966, 44.9% (260/601) and 33.2% (269/811) of

kangaroo meat samples imported to Japan from Australia for human consumption were contaminated with *Salmonella* spp. (Suzuki, Kawanishi et al. 1967). These levels are amongst the highest contamination rates reported to date. It is likely that in the 1950s and 1960s, following a sudden increase in the popularity of kangaroo meat (Pople and Grigg 1999), a limited range of hygiene standards were in place to minimise carcass contamination. Stricter industry regulations were not introduced until the 1970s and 1980s, along with the enforcement of Commonwealth approved state *Management Plans* (Pople and Grigg 1999). It is now a requirement that an AQIS inspector be present at each processing plant involved in the export of kangaroo meat (AQIS 1999). With improved hygiene practices now in place, more recent studies have reported decreased contamination of kangaroo carcasses compared to Suzuki et al. (1967) (Bensink, Ekaputra et al. 1991; Eglezos, Sofroni et al. 2007; Holds, Pointon et al. 2008). Despite the isolation of *Salmonella* spp. from kangaroo meat, there are no published reports of outbreaks of disease in Australia linked to the consumption of the product. Given the reported contamination rates in the aforementioned studies are higher than the observed prevalence of *Salmonella* in free-ranging kangaroos in this study, shooters need to be more vigilant during the evisceration process to avoid gut perforation. Processors also need to ensure that they have adequate hygiene standards in place, particularly where they produce meat for pet food or local human consumption and are not formally required by AQIS to undertake sampling for *Salmonella* culture.

The low, estimated prevalence of *Salmonella* spp. in the study population suggests that kangaroos pose no greater risk of zoonotic transmission than other livestock species in their natural environment. It is important to note that the comparison is

being made between rates of faecal shedding and not carcass contamination at the processor, which have different aetiologies. Prevalence is generally higher at the slaughterhouse due to stress, high stocking rates and carcass cross-contamination (D'Aoust 1989). Poultry constitute the most important animal reservoir of *Salmonella* (D'Aoust 1989). Infection in both the birds and their eggs varies depending on hygiene, feed, housing, geographic region and the bird's age. A study in Australia reported that *Salmonella* spp. were isolated from approximately 32% of whole egg, egg pulp and egg yolk samples from a poorly managed farm (Cox, Woolcock et al. 2002). Pigs are also considered common reservoirs of the organism and as a result, have become the focus of efforts to reduce herd infection rates and improve diagnostic tests, particularly in the Netherlands (van der Wolf, Elbers et al. 2001) An on-farm examination of healthy slaughter-age cattle and sheep in Australia demonstrated that dairy cattle were significantly more likely to shed *Salmonella* in faeces than pasture beef cattle, mutton sheep and prime lambs (Vanselow, Hornitzky et al. 2007). However, studies have demonstrated that less than 8% of cattle, pigs and sheep shed *Salmonella* in their faeces (Huston, Wittum et al. 2002; Mcevoy, Doherty et al. 2003; Fegan, Vanderlinde et al. 2004; Futagawa-Saito, Hiratsuka et al. 2008). These findings are very similar to the observed results in the population of kangaroos in this study.

3.4.3. *Salmonella* Serotypes Isolated from Kangaroos

Seven different *Salmonella* serotypes were isolated from the study population of kangaroos, including *Salmonella enterica* serovars Muenchen, Rubislaw, Kiambu, Lindern, Champaign, Saintpaul and II 42:g,t:- (previously *S.* ser. Fremantle). A number of these serovars have been associated with salmonellosis in humans,

although not related to the consumption of kangaroo meat. *Salmonella* serovars Muenchen and Saintpaul were among the top ten isolates from humans with salmonellosis in Australia between 1987 and 1992 . More significantly, *Salmonella* serovars Saintpaul and Muenchen were among the top five serotypes isolated in Western Australia from human infections (Murray 1994). Similar results were reported for 2005 (OzFoodNet Working Group 2007). Furthermore, *S.* ser. Kiambu was responsible for two outbreaks of food-borne salmonellosis in restaurants in Western Australia in the same year (OzFoodNet Working Group 2006). *Salmonella* ser. Rubislaw has been associated with food-borne salmonellosis in Germany (OzFoodNet Working Group 2006) and a lethal zoonotic infection of a three week old baby following contact with an infected pet reptile in the United Kingdom (Lehmacher, Bockemühl et al. 1995). As kangaroo meat consumption and contact with kangaroos provides a potential route for infection with *Salmonella* spp*.,* appropriate measures should be taken to maintain hygiene when cooking kangaroo meat, handling the animals or cleaning their living quarters.

A number of *Salmonella* serotypes isolated in this study have also been detected in livestock species and their meat products, suggesting that transmission of infection may occur between livestock and kangaroos. *Salmonella* serovars Muenchen, Kiambu and Saintpaul have been isolated from cattle and a number of other species, including sheep and horses (Murray 1994; Ward 2000; National Enteric Pathogen Surveillance Scheme 2006). *Salmonella* ser. Rubislaw has most commonly been isolated from goats, but has also been reported in cattle, beef and sheep meat. *Salmonella* serovars Champaign and Lindern have not been isolated from any other non-human animal species or meat since 1997 (National Enteric Pathogen Surveillance Scheme 1998;

1999; 2000; 2001; 2002; 2003; 2004; 2005; 2006; 2007; 2008). As cattle and sheep were the most abundant species at the livestock/wildlife interface in all study locations, cross-infection may have occurred with kangaroo poulations. Further research is required to determine the transmission dynamics between domestic livestock and kangaroo populations. This would require simultaneous studies to be undertaken in both kangaroos and ruminants found to be co-grazing on the same pastures.

3.4.4. Association between Rainfall and Quarter and the Prevalence of *Salmonella* in Kangaroo Faeces

The observed association between rainfall and the prevalence of *Salmonella* spp. isolated from kangaroo faeces suggests that there are seasonal fluctuations in the rate of shedding in kangaroos. These results support the work of Bensink et al. (1991) who reported higher levels of *Salmonella* contamination of feral pig carcasses in Australia during the wetter periods of the year and How et al. (1983), who noted that *Salmonella* infection in mammals in northern Western Australia peaked during the wet season. In contrast, Eglezos et al. (2007) reported a statistically significant relationship between increased rates of kangaroo carcass contamination at the processor and the summer months of the year. Hart et al. (1985) similarly demonstrated that the prevalence of faecal shedding in the quokka (*S. brachyurus*) peaked as high as 70 – 100% during summer. In light of these contrasting findings, care should be taken when extrapolating the results of such studies to kangaroos in their natural habitat. The statistical relationship in the study undertaken by Bensink et al. (1991) and Eglezos et al. (2007) was drawn between the route of carcass contamination at the processor and season only. It says nothing of the route of

infection in wild kangaroos and the region of Australia in which they were sampled. Scavenging for human food scraps and disruption of gut physiology in quokkas on Rottnest Island following a feed shortage in summer is likely to play a significant role in the increased faecal shedding of *Salmonella.* Western grey kangaroos on the mainland are unlikely to be exposed to such severe feed shortages because farming practices have modified feed availability.

Faecal prevalence of *Salmonella* spp. was highest in the April to June quarter in this study. This time of year represents mid autumn through until early winter and coincides with the time in which the first major rains fall in the southwest of WA. Whilst April, May and June do not necessarily receive the highest monthly rainfall for the year, they often experience the most dramatic change in rainfall, following dry summer months. As demonstrated by numerous studies, vegetation growth lags rainfall by 30 – 60 days (Roderick 1994; Chandrasekar, Sesha Sai et al. 2006; National Enteric Pathogen Surveillance Scheme 2008) and is likely to have resulted in an increased abundance of green feed in the April to June quarter. In grazing animals, faecal consistency becomes less formed in the winter months following increased rainfall, likely due to a combination of a sudden change in diet and increased exposure to gastrointestinal parasites (Larsen, Anderson et al. 1999; Damizadeh, Saghafian et al. 2001). A similar phenomenon is likely to occur in kangaroos. Indeed, faecal samples collected during May and June were often noted to be brighter green in colour and of an unformed nature. Previously they had been firm, dry and brown. Consistency returned to normal within one to two months. This may have contributed to the increase in faecal shedding of *Salmonella* spp. at this time. Studies have demonstrated that physical properties of feed in pigs can influence the rate of survival

of *Salmonella* spp. in the gastrointestinal tract (Karlsson, Pollott et al. 2004).Whilst not a classical example of diet related stress, the sudden abundance of green feed may have temporarily disrupted digestive physiology and altered the intestinal flora, such that the gut environment was more favourable for the multiplication of salmonellae.

Kangaroos and livestock can regularly be found co-grazing on pastures in the rural regions of Western Australia. The association between domestic livestock and grazing kangaroos may also account for the seasonality of *Salmonella* prevalence. Jones et al. (1999) noted that *Campylobacter* shedding in sheep was intermittent throughout the year, but increased during lambing, weaning and movement onto new pasture. Whilst farming practices vary from region to region, calving and lambing seasons are often timed to coincide with feed abundance. It is possible that latent *Salmonella* infections in domestic livestock become active at this time, leading to increased faecal shedding. Furthermore, loose faecal consistency and the presence of surface ground water may lead to more widespread environmental contamination. Professional Shooter A anecdotally reported that kangaroos in Capel were more likely to be found grazing on pasture during times when paddock feed was abundant. This coincided with the winter months, when environmental contamination was likely to be high. In the summer months, when pasture was drier, they tended to retreat to the National Park where competition for feed with livestock was reduced. As the WGK grazes between 5.9 and 9.8 hours per day (Mikkelsen, Naughton et al. 2004), the likely chance of ingestion of *Salmonella* from heavily contaminated pasture is high. Hence, the significant association between quarter and accumulated rainfall with prevalence is likely to be explained by the dual presence of increased numbers of kangaroos co-grazing with

livestock at a time when environmental contamination with *Salmonella* is at a maximum.

3.4.5. Association Between Location and the Prevalence of *Salmonella* in Kangaroo Faeces

The significantly higher prevalence of *Salmonella* in kangaroos sampled at Badgingarra was surprising given that annual rainfall is lower at this location compared to Nannup, Manjimup and Capel (Priddel 1986). This finding may be attributed to the geographical variation between collection locations and differences in climate, feed composition and ecology of the organism that are associated with this. With the exception of Eneabba, Badgingarra is the only sample collection site located in the Wheatbelt region of Western Australia, north of Perth. The remaining collection sites are located in or south of Perth. Whilst further studies are required to confirm the reason for this difference, the sudden abundance of green feed at Badgingarra, in the April to June quarter following rainfall, may have had a more profound effect on the digestive physiology of local kangaroos as pastures are generally drier throughout the year. In the Southwest of the state, rainfall extends over a greater number of months and average temperatures remain lower (Bureau of Meteorology 2009). The effect of a sudden change in feed composition may be diluted at Nannup, Manjimup and Capel as kangaroos are more accustomed to lush pasture. Although not statistically significant, it is interesting to note that the four most northern situated collection sites in this study reported the four highest prevalence levels.

4. A SURVEY OF WESTERN AUSTRALIAN KANGAROOS TO DETERMINE THE PREVALENCE OF *COXIELLA BURNETII*

4.1. Introduction

Livestock have traditionally been associated with transmission of *Coxiella burnetii* with the majority of infections occurring in agricultural workers involved with the farming or slaughter of ruminants (Bureau of Meteorology 2009). However, Derrick et al. (1939) proposed that marsupials were a potentially significant reservoir host for *C. burnetii* after demonstrating that bandicoots (*Isoodon torosus)* were susceptible to experimental infection, developing antibodies but no clinical signs (Garner, Longbottom et al. 1997). Pope et al. (1960) subsequently detected *C. burnetii* complement-fixing antibodies and agglutinins in a number of red (*Macropus rufus*) and grey (*Macropus major* Shaw) kangaroos. The organism was isolated in mice from the blood of one eastern grey kangaroo (*M. major*), suggesting that systemic infection does occur in kangaroos (Derrick, Smith et al. 1939). *Coxiella burnetii* was also isolated from 13 kangaroo ticks (*Amblyomma triguttatum*), four of which were found on goats and sheep (Pope, Scott et al. 1960). *Amblyomma triguttatum* is a 3-host tick and thus may be able to act as a vector between the different host species (Pope, Scott et al. 1960). The role of wildlife, in particular the kangaroo, in the maintenance and transmission of *C. burnetii* was highlighted in a recent study (Pope, Scott et al. 1960). An ELISA and quantitative PCR were developed to detect *C. burnetii* antibodies in serum and *C. burnetii* DNA in faeces from kangaroos, respectively. This was the first study investigating the role of macropods in transmission of *Coxiella* since 1960 and employed testing methods considered to be more sensitive than those adopted by Pope et al. (1960). Subsequent investigations are required to further define the role

that kangaroos play in the maintenance of *C. burnetii* and the transmission of infection to domestic reservoirs and people.

4.1.1. Aims of the Study

The aim of this study was to determine the seroprevalence of *C. burnetii* antibodies and the prevalence of faecal *C. burnetii* DNA in free-ranging kangaroos in Western Australia. The association between sex, age, location, accumulated rainfall and season (quarter) and both the seroprevalence of *C. burnetii* antibodies and prevalence of faecal shedding was determined.

4.2. Materials and Methods

4.2.1. Animal Data

Paired blood and faecal samples were collected from western grey kangaroos (WGKs) (*Macropus fuliginosus*) at twelve locations throughout the mid to southwest of Western Australia including: Badgingarra, Boyup Brook, Bridgetown, Capel, Eneabba, Greenbushes, Manjimup, Nannup, Northcliffe, Preston Beach, Scott River and Whiteman Park (*Figure 2.1*). For each sample, the location and date of collection was recorded as well as the sex and age of the animal. Shooters subjectively categorised kangaroos into subadult and adult age groups based on size and apparent sexual maturity (*Section 2.2.2.2)*. Subadults were considered to be those animals that had not yet reached mature body weight compared to adults, who were considered fully-grown. Pouch young serum was collected at two sites, Capel and Whiteman Park but it was not possible to collect faecal specimens at this age as the animals were too small.

4.2.2. Sample Collection

Serum samples were collected according to the methods described in *Section 2.2.2.4.* Briefly, 10 ml of blood was collected from each kangaroo either by cardiac puncture using a 10 ml Vacutainer® and 23G needle (Becton Dickinson, USA) or by the "free catch" technique shortly after the animal was deceased. The free catch method required the shooter to collect blood directly into a 10 ml serum collection tube once the carotid and jugular vessels were severed. Each kangaroo was tagged numerically when shot to ensure blood could be matched to the faecal sample taken during the evisceration procedure. Whole blood was refrigerated or stored in a chilled environment until serum could be separated. Once the clot retracted, serum was collected using a non-sterile, disposable pasteur pipette (201C, Copan Diagnostic Inc., USA), without centrifugation. Serum was stored in 2ml freestanding, screw-top tubes (2340-00, Scientific Specialists Inc, USA) at -20°C until required for testing.

Faecal samples were collected from kangaroos according to the method described in *Section 2.2.2.4.* Briefly, the abdomen and intestines were incised and between one and five balls were massaged from the distal colon into individual plastic specimen storage bags (Glad and Hercules supermarket brands) without coming into contact with the collector's hands. The identification number from the tag placed on the animal during blood collection was recorded on the faecal specimen bag, as was the animal's sex and age group. Faecal samples were placed into a freezer at -20°C within 24 hours of collection or stored at 4°C until this was possible.

4.2.3. Isolation of *Coxiella burnetii* Whole Genomic DNA from Faeces

Whole genomic DNA was extracted from each faecal sample using the MoBio PowerSoil™ DNA isolation kit (MO BIO, Calsbad, California, USA) according to Banazis (2009). A total of 0.2 g of faeces was added to the supplied bead-beating tubes, 60 µl Solution 'C1' was added and all tubes were mixed on a MO BIO vortex genie with 2 ml tube adaptor head for 30 seconds. Samples were then placed in a boiling water bath for five minutes, mixed on the vortex genie for one minute and then boiled for a further five minutes followed by vortex at maximum speed for 10 minutes. The PowerBead Tubes were then centrifuged at 10,000 x *g* for 30 seconds at room temperature. The supernatant was transferred into a clean 2 ml collection tube and 250 µl Solution C2 was added. The tube was placed on the vortex for 5 seconds then incubated at 4^{o}C for 5 min followed by centrifugation for 1 minute at 10,000 x *g*. The supernatant was transferred into another clean collection tube with 200 µl Solution C3. The tube was again placed on the vortex briefly for 5 seconds then incubated at 4^{o}C for 5 min followed by centrifugation for 1 minute at 10,000 x g. About 750 µl of the supernatant was transferred into a fresh tube with 1 ml Solution C4. Six hundred and seventy five microlitres of this mixture was loaded onto a spin filter and centrifuged at 10,000 x *g* for 1 minute. The above step was repeated to ensure all mixture was loaded on the spin filter. Next, 500 µl of Solution C5 was added and centrifuged at 10, 000 x *g* for 1 minute, the flow through was discarded and the spin filter was centrifuged at 10,000 x *g* for another minute. The spin filter was placed in a clean 2 ml tube and 100 µl of Solution 6 (Elution buffer) was added and then centrifuged at 10,000 x *g* for 30 sec. The spin filter was discarded and the DNA in the tube was ether used immediately or stored at 4°C until required.

4.2.4. Quantitative PCR Detection of *Coxiella burnetii* DNA Isolated from Faecal Samples

Quantitative PCR (qPCR) was undertaken on faecal samples from kangaroos according to the method described by Banazis et al. (2010) using two separate qPCR assays; one targeting the *IS1111a* element and one targeting the *JB153-3* sequence. The primer and probe sequences are shown in *Table 4.1* along with the final reaction concentration of the oligonucleotides. All reaction mixtures contained primers and probe at the concentrations indicated in *Table 4.1*, 7.5 µl UDG SuperMix (Invitrogen, Mount Waverley, Victoria, Australia), 3 mM (*JB153-3* assay) or 4.5 mM (*IS1111a* assay) magnesium chloride and 1 µl of template in a total volume of 15 µl. All samples were tested in duplicate on a Rotorgene 3000 (Corbett Life science, Mortlake, New South Wales, Australia) according to the following cycling parameters: One hold at 50°C for two minutes, a second hold at 95°C for two minutes followed by 45 cycles of 95°C for 20 seconds and 60°C (*JB153-3* assay) or 64°C (*IS1111a* assay) for 45 seconds. Two 'no template' controls (NTC) were included with every run. Each PCR run included a six-point standard curve comprising DNA extracted from Q-Vax™ vaccine (CSL, Parkville, Australia). The concentration of DNA from the Q-Vax™ vaccine was determined using a Nanodrop spectrophotometer and the number of *C. burnetii* genomes per microliter of cell suspension was calculated according to the molecular weight of the *C. burnetii* genome (Banazis, Bestall et al. 2010). The Rotorgene 3000 software was used to automatically select optimal cycle threshold cut-offs based upon the slope of the standard curve and the R^2 value. The user-defined DNA concentration of the standards was then used by the software to provide estimates of the DNA

concentration of the unknown samples. Results were expressed as genomes per microliter of DNA eluate.

Table 4.1 Primer and probe sequences used in qPCR to detect *Coxiella burnetii* DNA in kangaroo faeces

Name	5` to 3` sequence	5` label	3` label	Primer Concentration
*IS1111a*F	GTTTCATCCGCGGTGTTAAT	none	none	25 pmol
*IS1111a*R	TGCAAGAATACGGACTCACG	none	none	20 pmol
*IS1111a*P	CCCACCGCTTCGCTCGCTAA	6-FAM	BHQ-1	1.25 pmol
*JB153-3*F	TATTCGGCATCCCTTGGATA	none	none	15 pmol
*JB153-3*R	TTGTAACGCGCCACTATCTG	none	none	20 pmol
*JB153-3*P	TCACGCGCAATATTTGCAGCATG	6-FAM	BHQ-1	3.75 pmol

4.2.5. Detection of Antibodies to *Coxiella burnetii* in Serum from Kangaroos in Western Australia using an ELISA

Detection of *C. burnetii* antibodies in serum was undertaken using an antibody ELISA, according to Banazis et al. (2010). Nunc Maxisorp flat bottom microtitre plates (Nalge NUNC International, New York) were coated overnight at 4°C in a humid chamber with 100 µl of a solution containing equal proportions of reconstituted phase I and phase II *C. burnetii* antigens (Institut Virion/Serion GmbH, Germany) diluted 1 in 50 in carbonate/bicarbonate buffer (*p*H 9.6). Diluted antigen was discarded, excess solution was removed by tapping the plate on absorbent towel and the plates were inverted and dried at 37°C for 30 minutes. Each plate was blocked by addition 150 µl of Tris EDTA containing 0.05% Tween 20 (TEN-T, *p*H 8) and 3 %

w/v skim milk powder to each well followed by incubation for 60 minutes in a humid chamber at 37°C. The blocking solution was then discarded and the plates tapped on absorbent towel to remove excess buffer. Pooled sera from three kangaroo serum samples that had high optical densities in the ELISA and three samples that had low optical densities in the ELISA were used as 'positive' and 'negative' controls respectively. All serum samples were diluted 1 in 400 in TEN-T plus 1% w/v skim milk powder and allowed to stand at room temperature for 30 minutes. One hundred microliters of diluted control and test sera were added to four wells each and two wells each respectively and incubated at 37°C for 60 minutes in a humid chamber. The microtitre plates were then washed three times with TEN-T and 100 µl of rabbit anti-kangaroo IgG heavy and light chains (Bethyl Laboratories Inc., Montgomery, Texas, USA) diluted 1 in 500 in TEN-T plus 1% w/v skim milk powder was added to all wells and incubated in a humid chamber at 37°C for 60 minutes. Plates were washed as described previously and 100 µl of donkey anti-rabbit-HRP (Bethyl, Montgomery, Texas, USA) diluted 1 in 4,000 in TEN-T plus 1% w/v skim milk powder was added to all wells and then the plates were incubated at 37°C for 60 minutes in a humid chamber. The microtitre plates were washed a final time as described previously and 100 µl of TMB substrate (Pierce, Quantum Scientific, Murrarie, Queensland, Australia) was added to all wells and incubated at room temperature for 15 minutes before the reaction was stopped by adding 100 µl of 1M H_3PO_4 to each well. The plates were read using a BioRad Microplate Reader 6800 (BioRad, Regents Park, New South Wales, Australia) and the final optical density (OD) of each well was determined by subtracting the OD_{570nm} from the OD_{450nm}. The OD values of test samples were converted to a percentage of the mean positive control

OD's ('PP') from the same plate and all samples with values equal to or greater than 40% were classified as positive.

4.2.6. Environmental Data

Daily rainfall data was obtained from the Bureau of Meteorology (BOM) for weather stations located closest to the sample collection sites (*Table 4.2)*. Accumulated rainfall was calculated for the preceding 30 days and 60 days at each site for each date of collection. These time periods were chosen as they are consistent with the lag in Normalised Difference Vegetation Index (NDVI) following rainfall, noted in numerous studies (Roderick 1994; Chandrasekar, Sesha Sai et al. 2006; Barnesa, Mortona et al. 2007). The NDVI is an index used to monitor vegetation growth derived from satellite data. Rainfall records were not obtained for Whiteman Park and so samples from this location were excluded from all analyses incorporating rainfall. Accumulated rainfall in the preceding 30 days (RainCat30) and 60 days (RainCat60) were grouped into 4 categories: <25 mm, 25 – 49 mm, 50 – 99 mm and ≥100 mm; and <50 mm, 50 – 99 mm, 100 – 199 mm and ≥200mm, respectively. Data were also aggregated based on the quarter of the year in which they were collected: Q1=Jan-Mar; Q2=Apr-Jun; Q3=Jul-Sep; Q4=Oct-Dec.

Table 4.2 Bureau of Meteorology (BOM) weather station from which rainfall data was obtained for each sample collection site

Sample Collection Site	BOM Weather Station Number
Preston Beach	9679
Scott River	9926
Northcliffe	9590
Nannup	9585
Manjimup	9573
Greenbushes	9552
Eneabba	8225
Badgingarra	9037
Capel North	9992
Bridgetwon	9510
Boyup Brook	9504

4.2.7. Data Analysis

A generalised linear model which assumed a binomial distribution for the presence of *C. burnetii* was fitted to the data (both ELISA and qPCR) to determine whether there was an associatiom with sex, age, quarter or rainfall category (Damizadeh, Saghafian et al. 2001). The agreement between the two tests was assessed by calculating the Kappa statistic using the Statistics Package for Social Sciences (SPSS v.15) (SPSS Corporation, USA). The 95% confidence intervals surrounding estimated seroprevalence and prevalence proportions were calculated using the Adjusted Wald Method or Modified Wald Interval as it provides best coverage for the specified interval when sample sizes are small (McCullagh and Nelder 1989). The CHI-square test was used to determine whether any two proportions were significantly different from each other, unless one of the cell values was less than five, in which case

Fisher's Exact test was used at a 95% confidence limit. A separate analysis of the data from Capel was also undertaken because it was the only location where a large number of samples were collected across all rainfall and quarter categories as well as sex and age groups.

4.3. Results

4.3.1. Distribution of Samples Across Sex, Age, Rainfall and Quarter Categories for all Collection Locations

A total of 1017 serum and 990 faecal samples were collected from WGKs across twelve locations throughout the mid to southwest of Western Australia. The number of samples collected at each site ranged from 12 to 281 specimens. The sex of each kangaroo sampled was determined and recorded for 997 individuals (*Table 4.3*). The number of males and females were well distributed across all sample collection sites except Scott River, which had only 12 samples in total (*Table 4.3*). Out of the 760 samples where age was recorded, 26 were pouch young, 60 were subadults and the remainder were adults. Age was not consistently recorded for 227 animals harvested in Badgingarra, Preston Beach and Eneabba. Kangaroos were sampled in all accumulated rainfall (*Table 4.4)* and quarter (*Table 4.5*) categories in Capel only. Samples from the remaining collection locations were not as well distributed.

Table 4.3 The age and sex of kangaroos sampled at each study location

Location	Sex			Age				Total
	Female	Male	Unknown	Adult	Sub-adult	Pouch Young	Unknown	
Badgingarra	60	81	0	35	0	5	101	141
Boyup Brook	41	45	0	83	0	3	0	86
Bridgetown	24	30	0	52	0	0	0	54
Capel	100	175	6	233	24	23	1	281
Eneabba	55	50	0	13	0	4	88	105
Greenbushes	14	10	0	22	0	2	0	24
Manjimup	40	73	0	109	0	4	0	113
Nannup	20	14	0	26	0	8	0	34
Northcliffe	8	32	0	40	0	0	0	40
Preston Beach	49	26	0	22	0	6	47	75
Scott River	1	11	0	12	0	0	0	12
Whiteman Park	12	20	0	25	2	5	0	32
Total	**424**	**567**	**6**	**674**	**26**	**60**	**237**	**997**

Table 4.4 Number of samples collected in each accumulated rainfall category at each location

30 Day Accumulated Rain Category (RainCat30)	**< 25mm**	**25-49 mm**	**50-99 mm**	**≥ 100mm**
Badgingarra	60	53	0	28
Boyup Brook	20	66	0	0
Bridgetown	0	0	27	27
Capel	46	68	59	108
Eneabba	61	0	28	16
Greenbushes	0	0	0	24
Manjimup	31	0	61	21
Nannup	0	34	0	0
Northcliffe	0	40	0	0
Preston Beach	20	0	28	27
Scott River	0	0	0	0
Whiteman Park	0	0	0	0
Accumulated 60 Day Rain Category (RainCat60)	**< 50mm**	**50-99 mm**	**100-199 mm**	**≥ 200mm**
Badgingarra	113	0	0	28
Boyup Brook	35	20	31	0
Bridgetown	0	0	0	54
Capel	46	127	62	46
Eneabba	35	54	16	0
Greenbushes	0	0	0	24
Manjimup	31	32	50	0
Nannup	0	34	0	0
Northcliffe	0	0	40	0
Preston Beach	20	0	28	27
Scott River	0	0	0	0
Whiteman Park	0	0	0	0

Table 4.5 Number of samples collected at each location in each quarter

Location	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec
Badgingarra	9	75	28	29
Boyup Brook	0	66	0	20
Bridgetown	0	27	27	0
Capel	23	93	103	62
Eneabba	26	35	44	0
Greenbushes	0	0	24	0
Manjimup	31	32	21	29
Nannup	0	34	0	0
Northcliffe	0	0	0	40
Preston Beach	20	55	0	0
Scott River	0	0	0	12
Whiteman Park	0	0	32	0

4.3.2. Association Between Location and the Seroprevalence of *Coxiella burnetii* in Kangaroos in Western Australia

The overall seroprevalence of *C. burnetti* in 1017 kangaroos across all twelve locations was 24.1% (21.6 – 26.8). Seroprevalence estimates for each location are displayed in *Table 4.6.* Location had a significant effect on the estimated seroprevalence of *C. burnetii* ($p<0.001$). The estimated seroprevalence at Capel was significantly lower than all other sampling locations (10.7%, 7.5 – 14.9) ($p<0.005$) except Bridgetown, Northcliff, Nannup and Scott River. Conversely, the estimated seroprevalence at Whiteman Park (56.7%, 39.2 – 72.6) and Preston Beach (48%, 37.1 – 59.1) was significantly higher ($p<0.01$) than all other sampling locations except Boyup Brook, Scott River and Greenbushes.

Table 4.6 Estimate of C*oxiella burnetii* seroprevalence for kangaroos from each sampling location

Location	Seroprevalence (%)		95% CI
Capel	10.7	a	7.5, 14.9
Bridgetown	13.0	ab	6.1, 24.7
Northcliffe	17.5	abc	8.4, 32.3
Nannup	20.6	abcd	10.1, 37.1
Manjimup	23.0	bc	16.2, 31.6
Scott River	25.0	abcde	8.3, 53.9
Badgingarra	27.0	bcd	20.3, 34.8
Eneabba	27.6	bcd	19.9, 36.9
Boyup Brook	34.9	cde	25.6, 45.4
Greenbushes	45.8	de	27.9, 64.9
Preston Beach	48.0	e	37.1, 59.1
Whiteman Park	56.7	e	39.2, 72.6

abcde *Different letters indicate a statistical difference in seroprevalence between locations ($p<0.05$)*

4.3.3. Association Between Accumulated Rainfall, Quarter, Age and Sex and the Seroprevalence of *Coxiella burnetii* in Kangaroos from all Collection Locations

Accumulated rainfall over the 60 days preceding sample collection (RainCat60) was significantly associated with seroprevalence (p=0.034) whilst RainCat30 was not (p=0.427). The estimated seroprevalence levels for each location by RainCat30 and RainCat 60 categories are presented in *Table 4.7* and *Table 4.8,* respectively. There was highly significant interaction between both RainCat30 and RainCat60 with location (p=<0.001), indicating that differences in the number of seropositive animals between rainfall categories, varied with location. At Manjimup and Capel, seroprevalence increased significantly as accumulated rainfall over the 60 days prior to sample collection increased (p<0.05). At Eneabba, seroprevalence decreased significantly with increasing rainfall (p<0.05). As samples were not collected in all rainfall categories at all locations, this association was often difficult to interpret.

Quarter was significantly associated with seroprevalence (p=0.005), with seroprevalence in kangaroos lowest in the 4th quarter (Oct – Dec) than all other quarters. There was no significant difference between the seroprevalence of kangaroos in different categories of age and sex across the twelve sample collection sites.

Table 4.7 *Coxiella burnetii* seroprevalence for each RainCat30 category in all sample collection locations

Location	< 25 mm	95% CI	25 – 49 mm	95% CI	50 – 99 mm	95% CI	≥ 100 mm	95% CI
Badgingarra	30.0[a]	18.5, 41.6	32.1[a]	19.6, 44.6	*NC*	*NC*	10.7[b]	0, 22.1
Boyup Brook	5.0[a]	0, 14.6	43.9 [b]	31.9, 55.9	*NC*	*NC*	*NC*	*NC*
Bridgetown	*NC*	*NC*	*NC*	*NC*	18.5[a]	3.8, 33.2	7.4[a]	0, 17.2
Capel	2.2[a]	0, 6.1	11.8[b]	4.2, 19.4	8.5[ab]	1.4, 15.6	14.8[b]	8.1, 21.5
Eneabba	41.0[a]	28.7, 53.3	*NC*	*NC*	10.7[b]	0, 22.1	6.3[b]	0, 18.1
Greenbushes	*NC*	*NC*	*NC*	*NC*	*NC*	*NC*	45.8	25.8, 65.8
Manjimup	3.2[a]	0, 9.3	*NC*	*NC*	23.0[b]	12.4, 33.6	52.4[c]	31.0, 73.8
Nannup	*NC*	*NC*	20.6	7.1, 34.1	*NC*	*NC*	*NC*	*NC*
Northcliffe	*NC*	*NC*	17.5	5.7, 29.3	*NC*	*NC*	*NC*	*NC*
Preston Beach	25.0[a]	6.0, 44.0	*NC*	*NC*	77.8[b]	62.1, 93.5	25.9[a]	9.4, 42.4

NC *Samples not collected in rainfall category for given location*

abc *Different letters indicate a statistical difference in seroprevalence between RainCat30 categories for each collection location ($p<0.05$)*

Table 4.8 *Coxiella burnetii* seroprevalence for each RainCat60 category in all sample collection locations

Location	< 50 mm	95% CI	51 – 99 mm	95% CI	100 – 199 mm	95% CI	≥ 200 mm	95% CI
Badgingarra	31.0[a]	22.6, 39.4	*NC*	*NC*	*NC*	*NC*	10.7[b]	0, 22.1
Boyup Brook	60.0[a]	43.7, 76.3	5.0[b]	0, 14.6	25.8[c]	10.5, 41.1	*NC*	*NC*
Bridgetown	*NC*	*NC*	*NC*	*NC*	*NC*	*NC*	13.0	4.0, 22.0
Capel	2.2[a]	0, 6.1	10.2[b]	4.9, 15.5	6.5[ab]	0.4, 12.6	26.1[c]	13.4, 38.8
Eneabba	40.0[a]	23.7, 56.3	25.9[a]	14.3, 37.5	6.3[b]	0, 18.1	*NC*	*NC*
Greenbushes	*NC*	*NC*	*NC*	*NC*	*NC*	*NC*	45.8	25.8, 65.8
Manjimup	3.2[a]	0, 9.3	34.4[b]	17.9, 50.9	28.0[b]	15.7, 40.3	*NC*	*NC*
Nannup	*NC*	*NC*	20.6	7.1, 34.1	*NC*	*NC*	*NC*	*NC*
Northcliffe	*NC*	*NC*	*NC*	*NC*	17.5	5.7, 29.3	*NC*	*NC*
Preston Beach	25.0[a]	6.0, 44.0	*NC*	*NC*	77.8[b]	62.1, 93.5	25.9[a]	9.4, 42.4

NC *Samples not collected in rainfall category for given location*

abc *Different letters indicate a statistical difference in seroprevalence between RainCat60 categories for each collection location ($p<0.05$)*

4.3.4. Results of Testing Faeces for the Presence of *Coxiella burnetii* DNA Using Quantitative Polymerase Chain Reaction

Coxiella burnetii DNA was detected in 4.1% (3.1-5.6; n=990) of faecal samples. The individual prevalence estimates are listed in *Table 4.9* for each location. There was no significant difference in the proportion of faecal samples that were positive using qPCR between collection locations.

Table 4.9 Proportion of qPCR-positive faecal samples in kangaroos from each sample collection location

Location	Proportion (%)	95% Confidence Interval
Nannup	0.0	0, 8.8
Greenbushes	0.0	0, 12.1
Scott River	0.0	0, 21.6
Preston Beach	1.3	0, 7.9
Eneabba	2.9	0.6, 8.4
Whiteman Park	3.3	0, 18.1
Manjimup	3.5	1.1, 9.0
Bridgetown	3.7	0.3, 13.3
Capel	3.9	2.1, 7.2
Badgingarra	4.3	1.8, 9.2
Northcliffe	7.5	1.9, 20.6
Boyup Brook	9.3	4.6, 17.5

4.3.5. Association Between Accumulated Rainfall, Quarter, Age and Sex and the Seroprevalence of *Coxiella burnetii* in Kangaroos from Capel, Western Australia

The estimated seroprevalence of *C. burnetii* in kangaroos from Capel (n=281) was 10.7% (7.5 – 14.9). Accumulated rainfall in the 60 days preceding sampling (RainCat60) was significantly associated with seroprevalence (p=0.013). Seroprevalence was higher in kangaroos sampled during RainCat60 ≥200mm category compared to kangaroos sampled during the remaining rainfall RainCat60 categories (p=0.013) (*Table 4.8)*. The seroprevalence was also significantly higher in kangaroos sampled during the 50 – 99 mm category compared to kangaroos sampled during the < 50mm category. Accumulated rainfall in the preceding 30 days (RainCat30), quarter, sex and age had no significant effect on the estimated seroprevalence of *C. burnetii* antibodies in kangaroos at Capel.

4.3.6. Association Between Accumulated Rainfall, Quarter, Age and Sex and the Prevalence of *Coxiella burnetii* DNA in Kangaroo Faeces in Capel, Western Australia

A total of 3.9% (2.1-7.2) of faecal samples collected from kangaroos in Capel were found to contain *C. burnetii* DNA using the qPCR. There was no significant difference between the shedding rates in kangaroos in different categories of age, sex, quarter and rainfall.

4.3.7. Correlation Between Quantitative Polymerase Chain Reaction and Enzyme-Linked Immunosorbent Assay Results

There was poor agreement between the qPCR and ELISA results (Kappa = 0.120). The probability of a faecal sample being positive for *C. burnetii* DNA using qPCR was more likely if the ELISA result was positive (OR = 7.1) (p=<0.001).

4.3.8. Correlation Between the Serological Status of Mother and Pouch Young

Serum samples were collected from 24 paired mother and pouch young. There was no significant correlation between the serological status of the pouch young and the mother (p=0.538). A total of five pouch young (20.8%) were seropositive for *C. burnetii*. There was no significant association between faecal shedding in the doe (qPCR) and the serological status of the young (ELISA) (p=0.208).

4.4. Discussion

4.4.1. Validity of Results

A significant number of serum samples were seropositive for *C. burnetii* suggesting that kangaroos in this study had previously been infected or were currently infected with *C. burnetti* at the time of harvesting. When interpreting these results, it is important to consider the reliability of the immunological assay used. Banazis et al. (2009) reported that the reproducibility of the ELISA could be further improved. In its development, selection of an appropriate cut-off point for positivity was difficult due to an absence of known negative samples and a lack of appropriate tests by which to confirm the positive nature of the controls. The strain of antigen used may not have been representative of the antigenic profile of *C. burnetii* found in Australian

marsupials (Agresti and Coull 1998) as regional differences have been found to exist in the activity of the antigenic strain (Banazis 2009). Isolation and characterisation of *C. burnetii* from Australian marsupials would further improve antigen selection. To minimise the effect that the variability of the antigen may have had on results, normalisation of the absorbance values was undertaken by expressing the test sample absorbance values as a percentage of the positive control absorbance mean from the same plate (Rodolakis, Bouzid et al. 2007). The possibility of cross-reactivity must also be considered (Banazis, Bestall et al. 2010). Whilst no evidence has been found in kangaroos to suggest this occurs, cross-reactivity between *C. burnetii* and other organisms has been documented in people, mice and rabbits (Lukacova, Melnicakova et al. 1996; Banazis 2009).

It is difficult to interpret the low prevalence of *C. burnetti* DNA detected in the faeces of kangaroos sampled in this study. The observed low prevalence most likely reflects the intermittent periods of shedding of *C. burnetti* observed in several studies (Guatteo, Beaudeaua et al. 2007; Rodolakis, Berri et al. 2007). Intermittent shedding significantly reduces the sensitivity of any technique designed to detect faecal organisms, including PCR. Commonly, the seroprevalence of antibodies against a given organism will be many times greater than the isolation/detection rate of the organism itself (La Scola and Raoult 1996; Yabsley and Pittman Noblet 2002; Rodriguez-Vivas, Albornoz et al. 2004), which appears to be the case in this study. It may also be possible that faecal shedding is not the primary route of transmission in kangaroos. In other animals it has been shown that *C. burnetii* is shed for a number of days to months in milk, urine and faeces, commonly following parturition (Berri, Couriau et al. 2001; Akter, Choudhury et al. 2007). Kangaroos are unlikely to

contribute significantly to environmental contamination through excretion of birth products as their quantity is small compared to domestic livestock species (Arricau Bouvery, Souriau et al. 2003).

The possibility that the positive qPCR samples resulted from contamination of kangaroo faeces by phase II *C. burnetii* cultured in the same laboratory was ruled out by targeting the JB153-3 gene which is deleted in Phase II strains, in addition to the IS1111a insertion sequence (Banazis, Bestall et al. 2010). Due to the highly persistent nature of *C. burnetii* and the ease in which the organism can be disseminated, this was a very important step in ensuring the validity of the test results. However, as JB153-3 targets a redundant gene that is not present in all wild-type strains, it is possible that the sensitivity of the test may have been decreased as a result (Banazis 2009; Banazis, Bestall et al. 2010). Despite the poor agreement between the PCR and ELISA results, the probability of a faecal sample being positive for *C. burnetii* DNA using qPCR was found to be higher when the ELISA result was positive. This observation suggests that the positive detection of DNA is more likely to be *C. burnetti* and not a non-specific cross-reaction.

4.4.2. Disagreement Between the Seroprevalence and Faecal Prevalence of *Coxiella burnetii* in Western Grey Kangaroos

The poor agreement between the ELISA and PCR results is not unexpected given the intermittent and seasonal nature of *Coxiella burnetii* shedding. This has been reported elsewhere (Yanase, Muramatsu et al. 1997; Gardon, Heraud et al. 2001; Guatteo, Beaudeaua et al. 2007; Rodolakis, Berri et al. 2007). The presence of seronegative kangaroos apparently shedding the organism in faeces, however, is worthy of

discussion and has been reported in other species. In one study, two ewes remained ELISA negative at two months following lambing, despite vaginal swabs persistently revealing *C. burnetii* DNA using PCR (Banazis 2009). In livestock, this could be explained by the fact that the bacteria were localised in the placenta or the uterus of an animal without inducing systemic antibodies (Berri, Couriau et al. 2001). In kangaroos, it is possible that animals were shedding the organism but had not yet developed a detectable IgG response. Alternatively, *C. burnetii* antibodies produced during the early stages of infection may have waned over time. Similar observations have been reported in cows, where detectable antibodies against *C. burnetii* disappeared within several months (Yanase, Muramatsu et al. 1997; Berri, Couriau et al. 2001). Experimental infection studies need to be undertaken to determine the extent and duration of the immunological response mounted by kangaroos to further explain these findings. Reactivation of latent *C. burnetii* infections have been described in other species (Grist 1959) hence it is possible that a kangaroo may act as a source of infection more than once in their lifetime. Despite anatomical and physiological differences in macropod reproduction, an investigation to determine whether parturition has any effect on the level of *C. burnetii* shedding in the kangaroo is warranted. It may be possible for parturition to increase shedding of the organism in excrements other than those associated with birth itself.

4.4.3. Significance of the Kangaroo as a Reservoir Host of *Coxiella burnetii* in Western Australia

The high level of seropositivity observed in this study supports the hypothesis of Derrick et al. (1939) who proposed that marsupials were a potentially significant reservoir host of *C. burnetii*. More significantly, these results provide evidence to

support Pope et al. (1960) and Banazis et al. (2009) who suggested that kangaroos were likely to play an important role in the maintenance and transmission of *C. burnetii*. Whilst domestic livestock have historically been considered the primary reservoir of the organism (Sidwell and Genhardt 1966; McKelvie 1980; Psaroulaki, Hadjichristodoulou et al. 2006), several studies have failed to conclusively demonstrate this relationship (Dane and Beech 1955; Garner, Longbottom et al. 1997; Gardon, Heraud et al. 2001). In a recent local survey of 124 *Bos indicus* cattle held at a feedlot in Vasse, 157 *B. taurus* cattle farmed in Pinjarra and 50 merino ewes (*Ovis ovis*) farmed at Murdoch University, Western Australia, all bovine and ovine samples were found to be negative using the complement fixation test (Banazis 2009). These results suggest that another reservoir of infection may exist in the local region. Given the absence of apparent infection in local domestic livestock (Banazis, Bestall et al. 2010), the demonstrated association between risk of infection in people and contact with wildlife (Gardon, Heraud et al. 2001; Banazis 2009) and the findings from this study, it is possible that kangaroos play a significant role in the maintenance of *C. burnetii* in Australia. Unlike ruminants, kangaroos are unlikely to contribute to environmental contamination with *C. burnetii* through birth product expulsion due to anatomical and physiological differences associated with parturition, birth and the absence of a true placenta (Psaroulaki, Hadjichristodoulou et al. 2006). They are likely however, to excrete infectious particles in faeces. As isolation of the organism was not undertaken, future research is required to unequivocally confirm the presence of infection in these animals.

4.4.4. The Association Between Rainfall and Quarter and the Seroprevalence and Faecal Prevalence of *Coxiella burnetii* in Western Grey Kangaroos

It is difficult to interpret the association between rainfall and seroprevalence as samples were not collected in all rainfall categories at all locations and disease incidence cannot be evaluated by seroprevalence alone. In the individual analysis at Capel, the seroprevalence was significantly higher following increased periods of rainfall in the two months prior to sample collection. However, at Badgingarra and Eneabba, the highest seroprevalence was found among animals collected during the driest conditions. Given no significant association was found with fecal shedding, it is not possible to evaluate the effects of rainfall on the rate of infection with *C. burnetii*. This relationship requires further investigation as Gardon et al. (2001) reported a strong correlation between accumulated rainfall and disease incidence in people, with a lag period peaking at the second month. Whilst wetter conditions do not generally support aerosolization of infectious particles, these conditions do favor the presence of wildlife and arthropod vectors, whose activity is often dependent upon rainfall (Dawson 2002). Whilst the role of the tick in transmission of *C. burnetii* is still unknown, a positive correlation between disease and rainfall is frequently seen in arthropod-borne disease (Gardon, Heraud et al. 2001). Enright et al. (1971b) noted that the peak activity of *C. burnetii* in mid-winter coincided with the season of high tick activity and when the ticks were found to be harbouring the organism (Gardon, Heraud et al. 2001). Following isolation of *C. burnetii* from 13 kangaroo ticks (*Amblyomma triguttatum*), four of which were found on goats and sheep, Pope et al. (1960) suggested that the 3-host tick may be able to act as a vector between the different host species. Interestingly, engorged ticks were commonly found attached to

kangaroos shot by professional shooters involved in this study. Further studies investigating the potential role of ticks in the transmission of *C. burnetii* are warranted. This was not undertaken as part of this study due to its inclusion in another research investigation. The influence of rainfall on Q fever incidence may also berelated to increased reproduction in fast-breeding potential reservoir species, such as rodents and lagomorphs, following proliferation of feed (Burgdorfer, Pickens et al. 1963; Enright, Franti et al. 1971; Fiedler 1994). With increased shedding and aerosolization of *C. burnetii* associated with a greater presence of reservoir hosts, it is plausible that disease incidence rises following increased rainfall.

Quarter was observed to have a significant effect in Capel, suggesting that seroprevalence in kangaroos was lower in the Oct – Dec quarter than all other quarters. This timing coincides with the Spring/Summer period in Western Australia. Seasonal variation in seroprevalence has been reported elsewhere, with peak seroprevalence levels in livestock and wild animals being observed in the winter months in both North America and Japan (Enright, Franti et al. 1971; Webster, Lloyd et al. 1994). Whilst Banazis et al. (2010) found no significant evidence of *C. burnetii* shedding in cattle and sheep in Western Australia, the prevalence of detectable antibodies in wildlife and more significantly, the seasonal nature of infection, appears to be related to their association with infected livestock (Enright, Franti et al. 1971; Yanase, Muramatsu et al. 1997). Shedding of *C. burnetii* in ruminant species increases dramatically during parturition with seroprevalence peaking between one and three months later due to high levels of environmental contamination (Yanase, Muramatsu et al. 1997; Berri, Couriau et al. 2001). In Capel, cattle form the predominant livestock species and calving runs from late summer/early autumn until

late autumn/early winter (Professional Shooter A, Personal Communication, 24th August 2009). Given animals are shedding the organism, maximum environmental contamination is likely to occur toward the end of this period which coincides with the first major rains for the year (Enright, Franti et al. 1971). Kangaroos are likely to become infected at a similar time due to inhalation of the organism whilst co-grazing on lush green pastures with domestic ruminants. Epidemiological studies have tended to focus independently on the role of domestic livestock and wildlife species in the maintenance and transmission of *C. burnetii*. However, it is important that future research be aimed at characterising the relationship between the two cycles.

4.4.5. The Association Between Location and the Seroprevalence and Faecal Prevalence of *Coxiella burnetii* in Western Grey Kangaroos

The absence of a significant difference in the proportion of faecal samples positive for *C. burnetii* between collection locations was likely due to small sample sizes from a number of the study sites. As the assistance offered by professional shooters in this project was largely voluntary and caused some disruption to their normal routine, it was not possible to request larger numbers of samples to be collected. Future studies are required to collect a larger number of samples over a longer time frame to determine whether a significant difference does exist between geographic locations.

The statistical difference in estimated seroprevalence levels between collection locations is of great interest. As faecal shedding is likely to be seasonal and intermittent in nature, the seroprevalence may give a better indication of the risk of infection in local kangaroo populations. Whilst seroprevalence is only able to give an indication of prior exposure to the organism, it is likely to provide a crude indication

of the risk of exposure to the organism in the area. The high seroprevalence at Preston Beach is worthy of discussion because the property from which the kangaroos were harvested from was destocked approximately three years earlier. The property largely borders on residential land and there are no livestock within approximately two kilometres of the area. Persistence of the organism in the environment following shedding from infected livestock many years earlier may account for the high level of exposure to *C. burnetii* by kangaroos. Alternatively, it may be possible that wildlife reservoirs are able to maintain *C. burnetii* without the presence of livestock species. Given that the daily homes range of WGKs may be less than two kilometres (Bureau of Meteorology 2009), it is unlikely that those animals sampled in this study would have travelled far enough to reach stocked properties and come into contact with the organism in this manner (Arnold, Grassia et al. 1991).

The high seroprevalence and likely presence of *C. burnetii* at Whiteman Park may have potential public health implications. Kangaroo faeces can be found on grassed areas where families picnic and children play. Zoonotic infection may therefore occur through inhalation of infectious particles. A larger survey of local kangaroos in the area is required to confirm this finding.

4.4.6. The Association Between Sex and Age and the Seroprevalence and Faecal Prevalence of *Coxiella burnetii* in Western Grey Kangaroos

The sex of the kangaroos had no effect on the seroprevalence of *C. burnetii* or the proportion of faecal samples positive using the qPCR. This finding is in agreement with the work of Willeberg et al. (1980) who noted that there was no sex-associated difference in the seroprevalence of *C. burnetii* antibodies amongst cattle, horses or

cats. Whilst the same study found a greater number of male dogs had developed antibodies compared to female, the results were marginal and similar results have not been reported elsewhere. Despite female ruminants shedding higher amounts of the organism at parturition (Arnold, Grassia et al. 1991), evidence suggests that susceptibility to infection is the same for both sexes. The results from this analysis support this hypothesis in kangaroos. Interestingly, sex hormones may play a role in the pathogenesis of *C. burnetii*, with men being more symptomatic than women, despite equal seroprevalence (Tissot-Dupont, Raoult et al. 1992; Raoult, Tissot-Dupont et al. 2000; Berri, Couriau et al. 2001). A similar finding was noted in C57/BL6 mice clinically infected with *C. burnetii* (Raoult, Marrie et al. 2005). As no investigation was undertaken to determine the pathological changes associated within infection in kangaroos, it is unknown whether a similar response could be expected in the WGK.

Macropods do not possess the same reproductive anatomy and physiology as ruminants. Further research is required to investigate the nature of shedding in female kangaroos during parturition as well as the presence or absence of the organism in the pouch environment. Whilst no correlation existed between the ELISA and qPCR results of mother and young, five pouch young were seropositive for *C. burnetii*. It is possible that their seropositive status was a result of maternal transfer of immunity. Studies have demonstrated that maternal transfer is greatest following birth and again prior to exiting the pouch to prepare the young for life away from their mothers (Bell, Stephens et al. 1974; Adamski and Demmer 1999; Daly, Digby et al. 2007; Leone, Bechah et al. 2007). Alternatively, the seropositive pouch young may have mounted their own immune response after direct exposure to *C. burnetii*. Three of these

samples were collected from young in the month of July, whilst the remaining two were collected in September. Births generally occur between late spring and early summer in WGKs, with young exiting the pouch for the first time at 298 days (+/- 34 days) following birth and permanently at 323 days (+/- 23 days) (Deane, Cooper et al. 1990). Depending on when the young were born, it is possible that they could have ventured out of the pouch and been infected with *C. burnetii.*

Age had no effect on the seroprevalence of *C. burnetii* antibodies or the prevalence of faecal shedding in kangaroos from the twelve sample collection sites. This finding is in agreement with Willeberg et al. (1980), who reported no significant difference in antibody prevalence with age in dogs, cats, cattle and horses investigated in a study of serum submitted for blood chemistry analysis at the University of California, School of Veterinary Medicine, Davis. This relationship does however require further investigation. In the event that a greater number of samples had been collected, it is possible that a positive correlation may exist between the age of the animal and the likelihood of seropositivity, assuming that kangaroos maintain antibodies over a prolonged period of time. Whilst little is known of the nature of the immune response to *C. burnetii* in macropods, the frequency of antibodies in people have been found to increase with age, reflecting a progressive exposure to the antigen (Dawson 2002). Experimental infection studies in eastern grey kangaroos (*M. giganteus*) with Murray valley encephalitis demonstrated that antibodies were capable of persisting for at least six months, but titres decreased over this time (Ruiz-Beltrán, Herrero-Herrero et al. 2004). With no subsequent exposure to *C. burnetii,* it is possible that an antibody response may wane completely, allowing kangaroos to act as a source of infection more than once in their own lifetime. Interestingly, Leone et al. (2007) noted that

following clinical infection with *C. burnetii,* mature mice (14 months) had increased tissue bacterial burden and granuloma formation as well as defective responses to bacterial stimulation, compared to younger mice (1 month) (Kay, Young et al. 1985). Although kangaroos do not appear to exhibit clinical signs of disease, it is expected that seroprevalence should increase with age due to increased exposure to the antigen over time (Leone, Bechah et al. 2007). The relationship between age and infection in kangaroos requires further investigation as the inevitable age-based selection bias introduced through the kangaroo harvesting industry may have interfered with these results.

5. MONITORING THE SEROPREVALENCE OF ROSS RIVER VIRUS NEUTRALISING ANTIBODIES IN KANGAROOS IN WESTERN AUSTRALIA

5.1. Introduction

Ross River virus (RRV) is the aetiological agent of the most common mosquito-borne disease of humans in Australia (Done, Holbrook et al. 2002; Lindsay 2004; Ruiz-Beltrán, Herrero-Herrero et al. 2004; Russell and Kay 2004) and in particular, Western Australia (Gatton, Kay et al. 2005). In the southwest of WA, large outbreaks of RRV disease are recorded every three to four years in human populations, despite weather conditions and mosquito populations favouring an outbreak in some inter-epidemic years (Lindsay, Oliveira et al. 1996; Lindsay, Oliveira et al. 1997; Johansen, Broom et al. 2005; Lindsay, Breeze et al. 2005). It is hypothesized that vertebrate host populations, most likely macropods, are responsible for the maintenance and amplification of RRV (Kay, Hall et al. 1986; Aldred, Campbell et al. 1991; Vale, Spratt et al. 1991; Lindsay 1995; Johansen, Power et al. 2004; Old and Deane 2005). In the southwest of Western Australia, the western grey kangaroo (WGK) (*Macropus fuliginosus*) is suspected of playing a significant role in the epidemiology of RRV and may contribute to the cyclicity of the virus (Doherty, Standfast et al. 1971).

Research to date suggests that marsupials are more efficient amplifiers of RRV than eutherian mammals, which in turn are more efficient than birds (Kay, Hall et al. 1986; Kay and Aaskov 1989; Lindsay, Oliveira et al. 1997; Harley, Sleigh et al. 2001). Two

out of the seven successful attempts to isolate RRV from potential, non-human vertebrate hosts, were achieved in the agile wallaby (*Macropus agilis*) (Marshall and Miles 1984). Limited experimental studies have demonstrated that the eastern grey kangaroo (*Macropus giganteus*) and agile wallaby (*M. agilis*) are capable of developing a detectable viraemia (Doherty, Standfast et al. 1971). One such study undertaken by Kay, Hall et al. (1986) induced an antibody response in the eastern grey kangaroo (*M. giganteus*) within seven days of inoculation with the virus. As no experimental infection studies have been undertaken in the WGK, the role of this species as a reservoir of RRV remains unclear. In various published serosurveys, the seroprevalence of RRV in macropods of varying species has ranged from 11% through to 87.5% (Kay, Hall et al. 1986; Aldred, Campbell et al. 1991; Vale, Spratt et al. 1991; Old and Deane 2005). However, Lindsay (1995) found that thirty five percent of all WGKs sampled from a variety of locations in WA had neutralising antibodies to RRV, indicating that these animals are commonly infected with the virus and may play a role in transmission.

5.1.1. Aims of the Study

The aim of this study was to further define the role of the WGKs as a reservoir host of RRV and to assess whether surveillance in these animals could provide data to improve the accuracy of predictions of viral epidemics in human populations in Western Australia.

5.2. Materials and Methods

5.2.1. Animal Data

Blood samples were collected post-mortem from WGKs that were harvested by professional shooters at fifteen locations across the mid and southwest of Western Australia including: Capel, Myalup, Preston Beach, Eneabba, Badgingarra, Manjimup, Nannup, Bridgetown, Northcliffe, Boyup Brook, Balingup, Scott River, Greenbushes, Thomsons Lake and Whiteman Park (*Figure 2.1, Section 2.2.2.1*). For each sample, the location and date of collection was recorded as well as the sex and age of the animal. Shooters subjectively categorised kangaroos into subadult and adult age groups based on size and apparent sexual maturity. Pouch young were sampled at Capel, Myalup, Thomsons Lake and Whiteman Park.

5.2.2. Sample Collection

Whole blood was collected from each kangaroo either by cardiac puncture using a 9 ml Vacutainer® and 23G needle (Becton Dickinson, USA) or by the "free-catch" technique described in *Section 2.2.2.3*. Whole blood was refrigerated or stored in a chilled environment until serum could be separated. Once the clot retracted serum was collected using a non-sterile disposable pasteur pipette (201C, Copan Diagnostic Inc., USA), without the need for centrifugation. Serum was stored in 2 ml freestanding screw-top tubes (2340-00, Scientific Specialists Inc, USA) at - 20°C until tested.

5.2.3. Vero Cell Culture Maintenance

Vero cells, provided by the Arbovirus Surveillance and Research Laboratory (ASRL), Microbiology Department, University of Western Australia, were the only cell line used in this study. They were used to culture RRV stocks for use in the serum

neutralisation test (NT). Media and solutions were prepared as described in *Appendix F*. The Vero cell line was maintained in 225 cm^2 Falcon tissue culture flasks (Becton Dickinson, New Jersey, USA) using Growth M199 media (containing 5% foetal bovine serum; *Appendix F*).

5.2.4. Growth of Ross River Virus Stocks

Ross River virus strain DC 5692, representing the southwest (SW) genotype, was provided by the ASRL, Microbiology Department, University of Western Australia, for use in the NT. Ross River virus strain DC 5692 was isolated from the Peel region in Western Australia in 1999. All spent media was removed from a 225 cm^2 confluent flask of Vero cells. Next, 5 ml of Maintenance M199 media (containing 2% foetal bovine serum) followed by 100 µl of virus stock was added to the flask. The flask was incubated at 37°C in a 5% CO_2-enhanced atmosphere for 60 minutes and agitated every 10 minutes. A further 20 ml of Maintenance M199 media (2% FBS) was added to the flask before incubation under the same conditions. The flask was examined microscopically on a daily basis for cytopathic effect (CPE), evidenced by degenerative, morphological changes in the Vero cells. At four days post inoculation, 70-80% of the Vero cells had undergone CPE, indicating that the virus was ready for harvest.

5.2.5. Harvesting Ross River Virus Stocks

When 70-80% of the Vero monolayer inoculated with RRV had developed CPE, the supernatant was removed and placed into a 50 ml, sterile conical centrifuge tube. Two millilitres of foetal bovine serum was added to produce a final concentration of 10%

and the solution centrifuged at 4°C, 1360*g* for 10 minutes. The resultant supernatant was then aliquoted into pre-labelled, sterile Wheaton vials and stored at -70°C.

5.2.6. Calculating the Tissue Culture Infective Dose of Virus Stocks

The tissue culture infective dose ($TCID_{50}$) represents the amount of virus required to infect 50% of cell culture wells. In order to calculate the $TCID_{50}$ of the RRV stocks grown in and harvested from Vero cells, 25 µl of heat inactivated (mock serum) Maintenance M199 Media (2% FBS) was added to all wells of two 96-well Falcon Microtest^{TM} tissue-culture plates (#35 – 3072, Becton Dickinson, NJ, USA). An additional 25 µl was added to the final column (12), which acted as a cell control. The virus stock was serially diluted in eight 10-fold dilutions in Maintenance M199 media (2% FBS). Thorough mixing at each dilution was very important to ensure even dispersal of the virus. Twenty five microlitres of each virus dilution was added to twenty two duplicate wells (columns 1-11 of two rows), leaving column 12 as a control. The plates were incubated for one hour at 37°C in a 5% CO_2-enhanced atmosphere. Finally, 100 µl of Vero cells (containing approximately 1.5 x 10^6 cells) in Growth M199 media (5% FBS) was added to all wells and the plates incubated for five days at 37°C in a 5% CO_2-enhanced atmosphere. Following incubation, wells were microscopically examined for evidence of CPE and the number of wells per dilution with CPE was counted. The $TCID_{50}$ per 25 µl was calculated using software developed by Dr. Robert Coelen (The University of Western Australia), which is based on the formula of Reed and Muench (Lindsay 1995).

5.2.7. Serum Neutralisation Test

The serum neutralisation test (NT) used to detect RRV neutralising antibodies in kangaroo serum was adapted from Johansen et al. (2005). Twenty five microlitres of Blank M199 media (containing no foetal bovine serum) was added to each well in rows 2-8 of a sterile 96-well Falcon tissue-culture plate (Becton Dickinson, USA). Twenty microlitres of each serum sample was added to individual, sterile eppendorf tubes containing 180 µl of Blank M199 media (0% FBS) (1/10 dilution) and thoroughly mixed. The diluted serum samples were heat inactivated at 56°C for 30 minutes and allowed to cool. A total of 25 µl of each sample was added in duplicate to rows 1, 2 and 8 (serum control). Two-fold dilutions from row 2 through to row 7 were undertaken, discarding the excess 25 µl that remained. At this point, rows 1-7 contained a volume of 25 µl in each well, whilst the control row 8 contained a final volume of 50 µl. Next, RRV stock was serially diluted in Maintenance M199 media (2% FBS) such that a sufficient volume of working dilution containing 50 $TCID_{50}$s of infectious virus per 25 µl was produced. A total of 25 µl of the working solution was then added to all wells except control row 8. Test plates were then incubated at 37°C in a 5% CO_2-enhanced atmosphere for 60 minutes.

Virus-control assays were performed in conjunction with each NT to ensure the virus titre used was accurate. In a separate microtitre plate, 25 µl of Maintenance Media (2% FBS) was added to all wells in column 1-11, with 50 µl being added to column 12 as a cell control. Next, 25 µl of each virus dilution, including three, ten-fold dilutions beyond the working dilution, were added to eleven wells per dilution (columns 1 to 11). Virus-control plates were simultaneously incubated under the same conditions as each of the test plates for one hour. Following incubation, 100 µl of

Vero cells (containing approximately 1.5 x 10^6 cells) in Growth Media (5% FBS) were added to all wells of both the neutralisation and virus-control plates and incubated at 37°C in a 5% CO_2-enhanced atmosphere for five days. Each plate was then examined microscopically for evidence of CPE.

Neutralisation titres were expressed as the reciprocal of the highest serum dilution where CPE did not occur. Neutralisation titres ≥ 40 were considered positive. The assay was repeated if the infectious titre of virus used was less than or greater than 50-100 $TCID_{50}$s per 25 µl. A sample was retested if it produced different results compared to its duplicate.

5.2.8. Human Case Data

The annual number of reported cases of RRV disease in humans was obtained from the Mosquito-Borne Disease Control Section (MBDC), Environmental Health Hazards Unit, WA Department of Health for each individual Local Government and suburb/town listed in *Table 5.1*. Thomsons Lake was not listed as a suburb and was consequently classified as Beeliar, in Cockburn, which is the suburb where the lake is situated. Scott River was not classified under any one suburb/town and was simply listed in the Local Government of Augusta-Margaret River. The number of reported cases of RRV disease in humans in each month was also obtained at a Local Government and suburb level for Capel from July 2005 until June 2009.

Table 5.1 Regional, local government and suburb/town classification of each sample collection location according to the Australia Bureau of Statistics

Sample Collection Location	Region	Local Government	Suburb/Town
Badgingarra	Midwest (Central)	Dandaragan	Badgingarra
Balingup	Southwest	Donnybrook-Balingup	Balingup
Boyup Brook	Southwest	Boyup Brook	Boyup Brook
Bridgetown	Southwest	Bridgetown-Greenbushes	Bridgetown
Capel	Southwest	Capel	Ludlow
Eneabba	Midwest	Carnamah	Eneabba
Greenbushes	Southwest	Bridgetown-Greenbushes	Greenbushes
Manjimup	Southwest	Manjimup	Manjimup
Myalup	Southwest	Harvey	Myalup
Preston Beach	Perth Metropolitan (South)	Waroona	Preston Beach
Nannup	Southwest	Nannup	Nannup
Northcliffe	Southwest	Manjimup	Northcliffe
Scott River	Southwest	Augusta-Margaret River	
Thomsons Lake	Perth Metropolitan (South)	Cockburn	Beeliar

5.2.9. Mosquito Surveillance Data

Mosquito and arbovirus surveillance data was obtained from the Arbovirus Surveillance and Research Laboratory (ASRL), The University of Western Australia, for Capel only. Data was considered from two of the laboratory's long-term trap sites located nearest to where kangaroos were sampled, known as "CALM Village" and the "Stirling and Higgins Road intersection". The total number of mosquitoes, the total number of *Aedes camptorhynchus* mosquitoes, considered to play a significant role in RRV transmission in the region (Reed and Muench 1938), the number of isolates and the minimum infection rate (MIR) per 1000 mosquitoes was collated for each trap site by Dr. Cheryl Johansen (UWA) (Appendix G – J). Traps were monitored every two weeks by the ASRL. Data was not obtained for the remaining kangaroo sample collection locations because mosquito surveillance by the ASRL is restricted to selected sites along the coastal region of the WA's southwest, where RRV activity is most active. All other kangaroo sample collection locations were considered to be too far away from these sites for the mosquito data to be considered representative.

5.2.10. Environmental Data

Daily rainfall data was obtained from the Bureau of Meteorology (BOM) for weather stations located closest to the sample collection sites, except Whiteman Park (*Table 5.2)*. Accumulated rainfall was calculated for the 30 (RainCat30), 60 (RainCat60), 90 (RainCat90), 180 (RainCat180) and 360 (RainCat360) days preceding each date of sample collection at each site. The accumulated rainfall for each category was further grouped into 4 categories shown in *Table 5.3.* Data were also aggregated based on the Quarter of the year in which they were collected: Q1=Jan-Mar; Q2=Apr-Jun; Q3=Jul-Sep; Q4=Oct-Dec.

Table 5.2 Bureau of Meteorology weather station number from which rainfall data was obtained for each sample collection site

Sample Collection Site	BOM Weather Station Number
Preston Beach	9679
Scott River	9926
Northcliffe	9590
Nannup	9585
Manjimup	9573
Greenbushes	9552
Eneabba	8225
Badgingarra	9037
Capel North	9992
Bridgetown	9510
Boyup Brook	9504

Table 5.3 Rainfall categories for each accumulated rainfall variable

RainCat30	RainCat60	RainCat90	RainCat180	RainCat360
≤ 25mm	≤ 50mm	≤ 75mm	≤ 200mm	≤ 500mm
26 - 50mm	51 - 100mm	76 - 150mm	201 - 400mm	501 - 700mm
51 - 100mm	101 - 200mm	151 - 300mm	401 - 600mm	701 - 900mm
> 100mm	> 200mm	> 300mm	> 600mm	> 900mm

5.2.11. Data Analysis

A generalised linear model which assumed a binomial distribution was used to determine whether RRV NT results (positive/negative) were associated with location, accumulated rainfall, season, sex or age (Johansen, Broom et al. 2005). A similar, linear model with normally distributed residuals was fitted to the neutralisation titre data to determine whether there was any association between neutralising antibody titre and location, sex, age, quarter or rainfall category. Neutralisation titres were log transformed and represented in the following manner: Negative titre = 0; 40 = 1; 80 = 2; 160 = 3; 320 = 4; ≥ 640 = 5. Only adults and subadults were included in this analysis, because pouch young were considered unlikely to play a significant role in the transmission of RRV due to their limited exposure to mosquitoes. Additionally, pouch young were only sampled from a small number of collection locations and were possibly influenced by the immune status of their mothers. Samples with unknown age and sex were excluded. Whiteman Park was excluded from all analyses including rainfall, as rainfall data was not obtained.

The 95% confidence intervals for each estimated seroprevalence were calculated using the Adjusted Wald Method or Modified Wald Interval because it is the most accurate method of calculation when sample sizes are small (McCullagh and Nelder 1989). The CHI-square test was used to determine whether any two proportions were significantly different from each other. Pearson's correlation coefficient was calculated to determine the linear correlation between two continuous variables (*Figures 5.1, 5.2, 5.4, 5.5*). The t-test and one-way ANOVA were used to determine whether log transformed neutralising antibody titre means were significantly different

from one another. When rounding log transformed neutralising antibodies to the nearest whole number, the round half up convention was used.

Data from Capel was analysed separately because it was the only location for which mosquito surveillance data could be obtained and where sample collection was well distributed across rainfall, quarter, sex and age group categories. A nominal daily mosquito capture was calculated by dividing the total number of mosquitoes found in each trap by the number of days the trap had been set. It was then possible to accumulate the numbers of mosquitoes in the 14 days (1 collection period) preceding kangaroo sample collection and in the 42 days (3 collection dates) preceding kangaroo sample collection in Capel for both sites. Similar calculations were also carried out for the minimum infection rate of RRV per 1000 mosquitoes (MIR), except that the MIR was averaged rather than summed over the preceding days.

The reported cases of RRV disease in humans were converted to annual attack rates using population figures from the Australian Bureau of Statistics. Attack rates for each given year at each location were calculated from July through to the following June, which coincided with the arboviral season. These figures were correlated with the seroprevalence and average log transformed neutralising antibody titre in kangaroos over three different twelve-month periods; same time period (July – June), preceding the July by six months (Jan – Dec) and lagging the July by six months (Jan – Dec). Monthly attack rates were calculated at a Local Government and suburb/town level for Capel from July 2005 until June 2009. These attack rates were correlated with the average log transformed neutralising antibody titre and the average

seroprevalence for the same month, as well as one, two and three months both preceding and following kangaroo sample collection.

5.3. Results

5.3.1. Distribution of Samples Across Sex, Age, Rainfall and Quarter Categories for all Locations

A total of 2632 serum samples from WGKs from fifteen locations throughout the mid to southwest of Western Australia were tested using the NT. The number of samples collected at each site ranged from 16 at Balingup to 677 at Capel. The sex of each kangaroo sampled was determined and recorded for 2597 samples (*Table 5.4*). The number of males and females were well distributed across all sample collection locations except at Balingup and Manjimup, where males significantly outnumbered females ($p<0.05$). The majority of the 2346 samples with age recorded were considered adults with only 260 subadults and 102 pouch young sampled (*Table 5.4*). Pouch young samples were collected predominantly from Capel, with a small number collected at Myalup, Thomsons Lake and Whiteman Park. Age was not consistently recorded for 267 animals sampled by Professional Shooter C at Eneabba, Badgingarra, and Preston Beach. A further 19 samples did not have the age recorded at Thomsons Lake due to the fast-paced nature of the cull. No location had samples collected in all categories of the five accumulated rainfall variables, although samples from Capel were collected in all except the >900 mm category (RainCat360) (*Table 5.5*). Seven sites had samples collected in all quarters (*Table 5.6*).

Table 5.4 Distribution of samples in age and sex categories for all collection locations

	Age				Sex			
Location	**Adult**	**Subadult**	**Pouch Young**	**Unknown**	**Male**	**Female**	**Unknown**	**Total**
Badgingarra	259	13	0	76	195	153	0	**348**
Balingup	15	1	0	0	13	3	0	**16**
Boyup Brook	156	10	0	0	81	85	0	**166**
Bridgetown	53	0	0	0	29	24	0	**53**
Capel	544	50	83	0	298	267	12	**677**
Eneabba	62	20	0	105	86	101	0	**187**
Greenbushes	21	2	0	0	9	14	0	**23**
Manjimup	204	10	0	1	152	62	1	**215**
Myalup	137	4	14	0	80	63	12	**155**
Nannup	49	10	0	0	30	29	0	**59**
Northcliffe	121	0	0	0	79	42	0	**121**
Preston Beach	49	9	0	85	76	67	0	**143**
Scott River	105	11	0	0	65	51	0	**116**
Thomsons Lake	187	115	3	19	178	136	10	**324**
Whiteman Park	22	5	2	0	19	10	0	**29**
Total	**1984**	**260**	**102**	**286**	**1490**	**1107**	**35**	**2632**

Table 5.5 Number of samples collected in each accumulated rainfall category at each collection location

Rainfall Category (RainCat30)	≤ 25mm	26 - 50mm	51 -100mm	> 100mm
Badgingarra	109	53	104	82
Balingup	0	16	0	0
Boyup Brook	40	71	55	0
Bridgetown	0	0	27	26
Capel	241	144	153	139
Eneabba	101	0	70	16
Greenbushes	0	0	0	23
Manjimup	48	0	106	61
Myalup	41	0	20	94
Nannup	59	0	0	0
Northcliffe	81	40	0	0
Preston Beach	91	0	29	23
Scott River	20	56	18	22
Thomsons Lake	108	112	70	34
Whiteman Park	0	0	0	0
Rainfall Category (RainCat60)	**≤ 50mm**	**51 - 100mm**	**101 - 200mm**	**> 200mm**
Badgingarra	132	74	114	28
Balingup	0	0	16	0
Boyup Brook	53	58	55	0
Bridgetown	0	0	0	53
Capel	200	190	168	119
Eneabba	75	54	58	0
Greenbushes	0	0	0	23
Manjimup	48	31	44	92
Myalup	20	41	0	94
Nannup	30	29	0	0
Northcliffe	60	21	40	0
Preston Beach	49	42	29	23
Scott River	12	60	22	22
Thomsons Lake	108	126	33	57
Whiteman Park	0	0	0	0

Table 5.5 cont. Number of samples collected in each accumulated rainfall category at each location

Rainfall Category (RainCat90)	**≤ 75mm**	**76 -150mm**	**151 - 300mm**	**> 300mm**
Badgingarra	103	103	142	0
Balingup	0	0	0	16
Boyup Brook	33	105	28	0
Bridgetown	0	0	0	53
Capel	204	151	203	119
Eneabba	52	119	16	0
Greenbushes	0	0	0	23
Manjimup	48	31	25	111
Myalup	20	21	50	64
Nannup	30	29	0	0
Northcliffe	40	41	40	0
Preston Beach	20	76	47	0
Scott River	12	60	22	22
Thomsons Lake	172	47	77	28
Whiteman Park	0	0	0	0
Rainfall Category (RainCat180)	**≤ 200mm**	**201 - 400mm**	**401 - 600mm**	**> 600mm**
Badgingarra	128	220	0	0
Balingup	0	0	0	16
Boyup Brook	98	68	0	0
Bridgetown	0	26	27	0
Capel	287	248	112	30
Eneabba	148	39	0	0
Greenbushes	0	23	0	0
Manjimup	0	79	48	88
Myalup	20	30	85	20
Nannup	29	30	0	0
Northcliffe	0	0	60	61
Preston Beach	49	41	53	0
Scott River	0	38	34	44
Thomsons Lake	202	101	21	0
Whiteman Park	0	0	0	0

Table 5.5 cont. Number of samples collected in each accumulated rainfall category at each location

Rainfall Category (RainCat360)	≤ 500mm	501 - 700mm	701 - 900mm	> 900mm
Badgingarra	301	47	0	0
Balingup	0	0	0	16
Boyup Brook	99	67	0	0
Bridgetown	0	0	53	0
Capel	246	355	76	0
Eneabba	187	0	0	0
Greenbushes	0	0	23	0
Manjimup	0	0	140	75
Myalup	0	0	155	0
Nannup	0	29	30	0
Northcliffe	0	0	0	121
Preston Beach	0	29	114	0
Scott River	0	12	64	40
Thomsons Lake	8	171	97	48
Whiteman Park	0	0	0	0

Table 5.6 Number of samples collected at each location in each quarter

Location	Jan-Mar	Apr-Jun	Jul-Sep	Oct-Dec
Badgingarra	9	75	126	138
Balingup	0	0	0	16
Boyup Brook	20	60	66	20
Bridgetown	0	26	27	0
Capel	147	114	232	184
Eneabba	45	33	86	23
Greenbushes	0	0	23	0
Manjimup	48	31	61	75
Myalup	20	58	56	21
Nannup	30	29	0	0
Northcliffe	81	0	0	40
Preston Beach	20	52	0	71
Scott River	8	18	22	68
Thomsons Lake	0	172	104	48
Whiteman Park	0	0	29	0

5.3.2. Seroprevalence of Ross River Virus Neutralising Antibodies in Western Grey Kangaroos

The overall seroprevalence of RRV neutralising antibodies in WGKs sampled at fifteen collection sites in WA was 43.9% (42.0 – 45.8). Location was significantly associated with seroprevalence ($p<0.001$), with individual estimates for each sample collection site listed in *Table 5.7.* The seroprevalence of RRV antibodies was significantly higher in kangaroos harvested at Thomsons Lake reserve, (92.0%; 88.5 – 94.5), compared to all other remaining sites ($p<0.05$). The seroprevalence of RRV antibodies was significantly higher in kangaroos harvested at Capel (74.7%; 71.3 – 77.9), compared to the remaining thirteen sample collection sites ($p<0.05$). Seroprevalence was significantly lower at Badgingarra, Eneabba and Northcliffe than all other collection locations ($p<0.05$), except Nannup.

Table 5.7 Estimate of Ross River virus neutralising antibody seroprevalence for western grey kangaroos from each sampling location

Location	Seroprevalence (%)	95% CI
Eneabba	8.0[a]	5.7, 14.2
Badgingarra	9.1 [a]	5.6, 11.4
Northcliffe	10.7 [a]	6.3, 17.6
Nannup	11.9 [ab]	5.6, 22.8
Whiteman Park	24.1[b]	12.0, 42.4
Preston Beach	26.6[b]	20.0, 34.4
Myalup	27.1[b]	20.7, 34.6
Boyup Brook	30.7[b]	24.2, 38.1
Bridgetown	32.1[b]	21.0, 45.5
Manjimup	32.2[b]	26.2, 38.6
Greenbushes	34.9[b]	18.7, 55.2
Balingup	37.5[b]	18.4, 61.5
Scott River	42.2[b]	33.6, 51.3
Capel	74.7[c]	71.3, 77.9
Thomsons Lake	92.0[d]	88.5, 94.5

[abcd] *Different letters represent a significant difference in seroprevalence between locations*

5.3.3. Association Between Accumulated Rainfall and the Seroprevalence of Ross River Virus Neutralising Antibodies in Western Grey Kangaroos

A significant association was observed between seroprevalence and all accumulated rainfall variables considered in this study (RainCat30 – RainCat360) (p=<0.001). The deviance for each accumulated rainfall variable increased as the lag period moved from 30 to 360 days, indicating that accumulated rainfall over the preceding 360 days explained more of the variance in RRV seroprevalence than any other period of accumulated rainfall. The influence of rainfall on seroprevalence differed depending on where the samples were collected, as evidenced by the statistically significant interaction between rainfall and location (p<0.001). *Table 5.8* lists the seroprevalence at each location for each RainCat360 category. The seroprevalence in kangaroos sampled at Capel significantly decreased as rainfall increased within RainCat360 (p<0.05). Comparatively, the seroprevalence in kangaroos sampled at Thomsons Lake increased as rainfall increased within this category (p<0.05). For the remaining locations it was difficult to identify a clear association between seroprevalence and rainfall in the preceding period because samples were not well distributed across all rainfall categories.

Table 5.8 Ross River virus neutralising antibody seroprevalence for each RainCat360 category in all sample collection locations

	Seroprevalence (with 95% confidence intervals)							
Location	**≤ 500mm**	**95% CI**	**501 - 700 mm**	**95% CI**	**701 - 900 mm**	**95% CI**	**> 900mm**	**95% CI**
Badgingarra	7.2	4.3, 10.1	*NC*	*NC*	*NC*	*NC*	*NC*	*NC*
Balingup	*NC*	*NC*	*NC*	*NC*	*NC*	*NC*	35.4	13.4, 57.4
Boyup Brook	44.1[a]	34.5, 53.7	10.5[b]	3.2, 17.8	*NC*	*NC*	*NC*	*NC*
Bridgetown	*NC*	*NC*	*NC*	*NC*	32.1	19.6, 44.6	*NC*	*NC*
Capel	82 .0[a]	77.1, 86.9	71.0[b]	65.9, 76.1	64.4[b]	52.8, 76.0	*NC*	*NC*
Eneabba	10.9	14.0, 17.8	*NC*	*NC*	*NC*	*NC*	*NC*	*NC*
Greenbushes	*NC*	*NC*	*NC*	*NC*	33.7	15.3, 52.1	*NC*	*NC*
Manjimup	*NC*	*NC*	*NC*	*NC*	37.2[a]	29.8, 44.6	17.0[b]	9.0, 25.0
Myalup	*NC*	*NC*	*NC*	*NC*	24.6	17.9, 31.3	*NC*	*NC*
Nannup	*NC*	*NC*	21.1[a]	5.0, 37.2	6.3[a]	0, 14.7	*NC*	*NC*
Northcliffe	*NC*	*NC*	*NC*	*NC*	*NC*	*NC*	10.7	5.2, 16.2
Preston Beach	*NC*	*NC*	38.5[a]	20.7, 56.3	6.8[b]	0, 15.8	*NC*	*NC*
Scott River	*NC*	*NC*	8.3[a]	0, 24.0	54.0[b]	43.0, 65.0	31.2 [b]	16.9, 45.5
Thomsons Lake	91.3[a]	86.2, 96.4	94.4[ab]	91.1, 97.7	96.8[ab]	93.5, 100.0	99.9[b]	99.3, 100.0

NC *Samples not collected in given rainfall category*

[a, b] *Different letters represent statistically significant differences between quarter categories for a given location at the 5% level of significance.*

5.3.4. Association Between Quarter and Seroprevalence of Ross River Virus Neutralising Antibodies in Western Grey Kangaroos

A significant association was observed between quarter and seroprevalence ($p<0.001$) and once again, the nature of this association varied between locations ($p<0.001$) (*Table 5.9*). In kangaroos sampled from Capel, the seroprevalence decreased in each consecutive quarter from Jan-Mar through until Oct-Dec quarter. The proportion of seropositive animals was significantly higher in the Jan-Mar quarter than the Oct-Dec quarter ($p=0.003$). A similar pattern was noted at Thomsons Lake where the seroprevalence decreased between the second and fourth quarters and was significantly lower in Oct-Dec than in Apr-June ($p=0.033$). Samples were not collected from Thomsons Lake in the first quarter. Whilst not statistically significant, similar trends were noted in Badgingarra and Eneabba. At Manjimup, the seroprevalence in the Apr-Jun quarter was significantly lower than all other quarters ($p<0.005$), whilst at Boyup Brook seroprevalence in the Jul-Sep quarter was significantly highest ($p<0.02$).

Table 5.9 Ross River virus neutralising antibody seroprevalence (%) for each quarter in all sample collection locations

	Seroprevalence (with 95% confidence intervals)							
Location	**Jan-Mar**	**95% CI**	**Apr-Jun**	**95% CI**	**Jul-Sep**	**95% CI**	**Oct-Dec**	**95% CI**
Badgingarra	0[a]	0, 0.6[a]	11.4[b]	4.5, 18.3	8.9[bc]	3.4, 14.4	3.3[ac]	0, 7.0
Balingup	*NC*	*NC*	*NC*	*NC*	*NC*	*NC*	35.4	313.4, 57.4
Boyup Brook	0[a]	0, 0.4	24.8[b]	14.4, 35.2	49.8[c]	37.8, 61.8	10.0[ab]	0, 23.1
Bridgetown	*NC*	*NC*	26.9[a]	9.8, 44.0	37.0[a]	18.8, 55.2	*NC*	*NC*
Capel	85.1[a]	78.2, 90.1	74.7[ab]	65.3, 82.3	72.8[b]	66.2, 78.6	69.8[b]	62.3, 76.4
Eneabba	*NC*	*NC*	13.6[a]	0, 31.0	9.7[a]	0.3, 19.1	8.7[a]	0, 20.3
Greenbushes	*NC*	*NC*	*NC*	*NC*	33.7	15.3, 52.1	*NC*	*NC*
Manjimup	31.5[a]	19.2, 43.8	6.1[b]	0, 14.3	35.1[a]	23.9, 46.3	34.4[a]	24.4, 44.4
Myalup	20.0[a]	2.6, 37.4	22.5[a]	12.3, 32.7	30.9[a]	18.4, 43.4	23.3[a]	5.9, 40.7
Nannup	6.3[a]	0, 14.7	21.1[a]	5.0, 37.2	*NC*	*NC*	*NC*	*NC*
Northcliffe	9.9[a]	3.4, 16.4	*NC*	*NC*	*NC*	*NC*	12.5[a]	12.3, 22.7
Preston Beach	*NC*	*NC*	23.1[a]	13.6, 36.2	*NC*	*NC*	32.9[a]	22.6, 44.0
Scott River	62.5[a]	29.0, 96.0	22.1[a]	3.3, 40.9	39.3[a]	18.9, 59.7	45.0[a]	34.0, 56.0
Thomsons Lake	*NC*	*NC*	94.2[a]	89.5, 96.9	92.3[a]	85.4, 96.3	83.3[a]	70.2, 91.6

NC *Samples not collected in given rainfall category*

[a, b] *Different letters represent statistically significant differences between quarter categories for a given location at the 5% level of significance.*

5.3.5. Association Between Age and Sex and the Seroprevalence of Ross River Virus Neutralising Antibodies in Western Grey Kangaroos

The seroprevalence of RRV neutralising antibodies amongst subadult kangaroos was not statistically different from the seroprevalence amongst adult kangaroos when all locations were considered together (p=0.463). However, the analysis was complicated by the fact that very few subadults were sampled from the majority of collection sites. Comparison of the seroprevalence of RRV neutralising antibodies between the two age groups for each individual collection location (*Table 5.10*) demonstrated that the seroprevalence amongst adults was generally greater than in subadults. This was particularly true for Thomsons Lake and Capel where larger numbers of subadult kangaroos were sampled. No association was observed between seroprevalence of RRV neutralising antibodies and the sex of kangaroos.

Table 5.10 Seroprevalence of Ross River virus neutralising antibodies for each age group of kangaroos from all sample collection locations

	Seroprevalence (%)			
Location	**Adult**	**95% CI**	**Subadult**	**95% CI**
Badgingarra	8.1*	4.8, 11.4	0	0, 0.4
Balingup	40*	15.3, 64.7	0	0, 1.6
Boyup Brook	29.8	23.3, 36.3	20.0	0, 44.7
Bridgetown	32.1	19.6, 44.6	*NC*	*NC*
Capel	76.3*	71.6, 81.0	44.7	28.8, 60.6
Eneabba	9.7	2.3, 17.1	20.0	2.6, 37.4
Greenbushes	38.1*	17.3, 58.9	0	0, 1.2
Manjimup	34.9*	28.4, 41.4	0	0, 0.6
Myalup	27.7*	20.3, 35.1	0	0, 0.8
Nannup	15.3*	5.1, 25.5	0	0, 0.6
Northcliffe	10.7	5.2, 16.2	*NC*	*NC*
Preston Beach	25.3*	14.1, 36.5	0	0, 0.6
Scott River	35.1*	26.5, 43.7	0	0, 0.6
Thomsons Lake	98.4*	96.8, 100.0	67.4	53.7, 81.1

NC *Samples not collected for specified age group*

* *Seroprevalence in the given age group statistically higher (at the 5% level of confidence) compared to the second age group, at the same location.*

5.3.6. Correlation Between Seroprevalence and the Average Log Transformed Ross River Virus Neutralising Antibody Titre in Western Grey Kangaroos

There was a significant, positive correlation between the seroprevalence and the average log transformed RRV neutralising antibody titre for all samples collected from the fifteen locations (r=0.98, p<0.001) (*Figure 5.1*). Western grey kangaroos from Thomsons Lake reserve recorded the highest average log transformed neutralising antibody titre at 4.2 (p<0.001) out of all collection locations (*Table 5.11*). Kangaroos from Capel recorded the second highest average log transformed neutralising antibody titre at 2.5, compared to the remaining 13 sample collection sites (p<0.001). When rounded to the nearest whole number, these averages represented neutralising antibody titres of 320 and 160, respectively. The average log transformed neutralising antibody titres at Northcliffe, Badgingarra, Eneabba and Nannup were indicative of a negative result (<40), whilst kangaroos from all other collection locations recorded an average neutralisation titre of 40, representing a weak positive.

When considering only the positive samples, there was a significant, positive correlation between the seroprevalence and average log transformed neutralising antibody (r=0.83, p<0.001) (*Figure 5.2)*. Kangaroos from Thomsons Lake recorded the highest average positive result at 4.5 (4.4, 4.6), representing a maximum antibody titre of ≥ 640 (p<0.001). Capel recorded the second highest average positive titre of 3.3 (3.2, 3.5), which was statistically higher than all other collection locations (p<0.001) except Whiteman Park, Preston Beach, Scott River and Nannup. There was no significant difference between the antibody titres of the remaining locations.

Table 5.11 Seroprevalence and average log transformed Ross River virus neutralising antibody titres for all samples and positive samples only collected at each location (ranked according to mean log transformed antibody titre of all samples)

Location	Seroprevalence (%)	Log Transformed Antibody Titre (all samples)	95% CI	Log Transformed Antibody Titre (positive samples)	95% CI
Northcliffe	9.9	0.2 [a]	0.1, 0.3	1.5 [a]	1.1, 1.9
Badgingarra	8.3	0.2 [a]	0.1, 0.3	2.1 [ab]	1.6, 2.6
Eneabba	9.1	0.2 [a]	0.1, 0.3	2.1 [ab]	1.6, 2.7
Nannup	11.9	0.3 [ab]	0.1, 0.5	2.6 [abcde]	1.7, 3.4
Myalup	27.0	0.6 [b]	0.4, 0.7	2.1 [ab]	1.7, 2.4
Boyup Brook	30.1	0.8 [bc]	0.6, 1.0	2.5 [bc]	2.1, 2.8
Bridgetown	32.1	0.8 [bc]	0.4, 1.1	2.4 [bc]	1.9, 2.9
Balingup	37.5	0.8 [bc]	0.2, 1.3	2.0 [abc]	1.3, 2.7
Preston Beach	26.6	0.8 [bc]	0.5, 1.0	3.0 [cde]	2.5, 3.4
Whiteman Park	26.7	0.8 [bcd]	0.3, 1.4	3.1 [bcde]	2.0, 4.3
Manjimup	31.6	0.9 [c]	0.7, 1.1	2.7 [bcd]	2.3, 3.0
Greenbushes	34.8	1.0 [bcd]	0.3, 1.6	2.8 [bcd]	1.7, 3.8
Scott River	42.2	1.4 [d]	1.0, 1.7	3.2 [de]	2.8, 3.6
Capel	74.9	2.5 [e]	2.4, 2.7	3.3 [e]	3.2, 3.5
Thomsons Lake	92.0	4.2 [f]	4.0, 4.3	4.5 [f]	4.4, 4.6

[abcdef] *Different letters indicate a significant different in average log transformed neutralising antibody titres between locations for either "all samples" or "positive samples only".*

Figure 5.1 Correlation between the seroprevalence and average log transformed Ross River virus neutralising antibody titre for all samples collected at each location

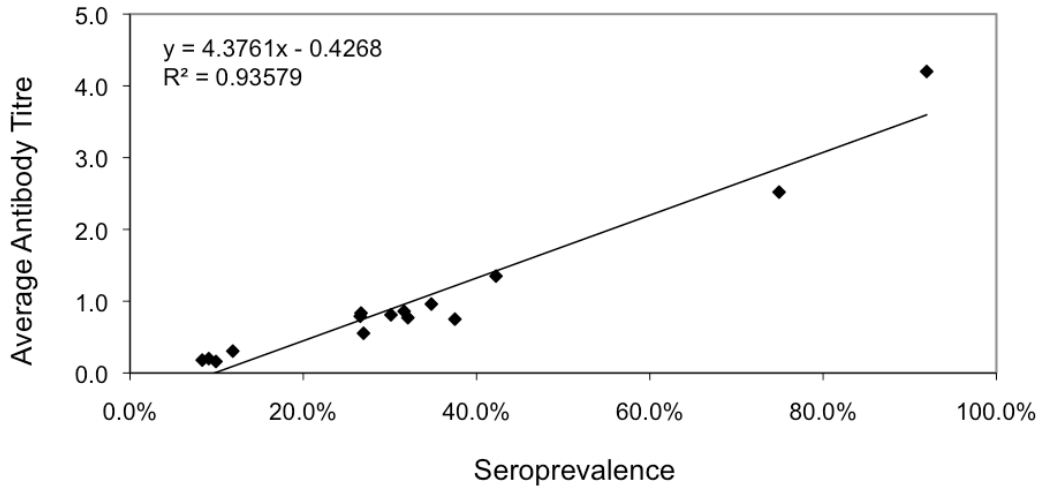
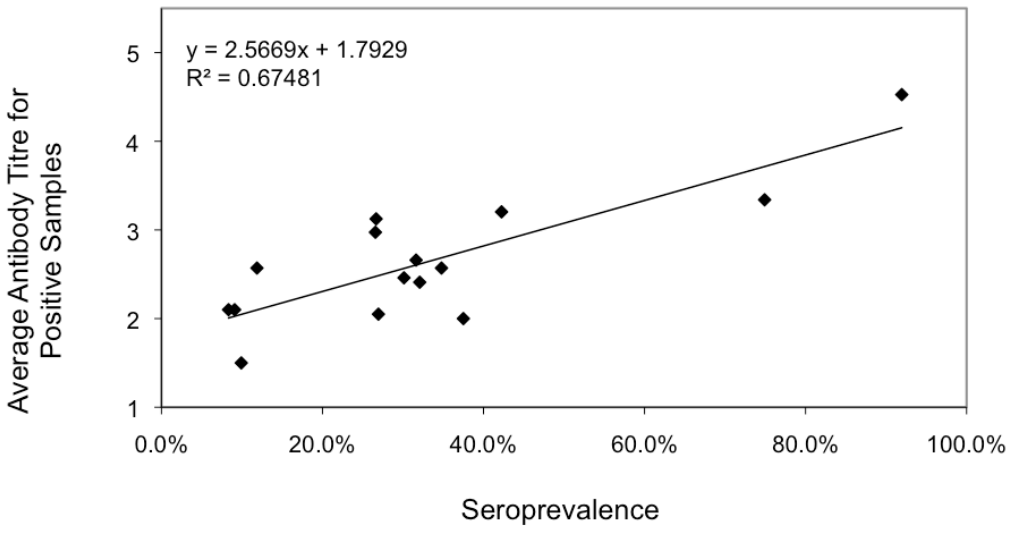


Figure 5.2 Correlation between the seroprevalence and average log transformed Ross River virus neutralising antibody titre for all positive samples collected at each location



5.3.7. Temporal Changes in Seroprevalence and the Average Ross River Virus Neutralising Antibody Titre in Western Grey Kangaroos

A significant temporal change in the seroprevalence of RRV neutralising antibodies in kangaroos sampled at Preston Beach, Capel and Thomsons Lake was reported over the duration of the study. Over five separate collections, between June 2007 and May 2008, the average seroprevalence at Preston Beach was 19.0% (8.5 – 31.2). In December 2008, the seroprevalence increased significantly to 78.0% (54.3 – 91.5) (p<0.001). No sample collection was undertaken between May and December 2008. At Thomsons Lake, seroprevalence decreased significantly from 94.2% in the Apr-Jun quarter to 83.3% in the Oct-Dec quarter of 2006 (p=<0.05). In Capel, the average seroprevalence also decreased significantly from 78.5% in 2007 (72.4 – 82.9) to 68.5% (59.9 – 76.1) in 2008 (p=0.030) (*Figure 5.3)*.

The average RRV neutralising antibody titre in kangaroos also decreased over the duration of the study at a number of collection locations. At Capel, the average log transformed neutralising antibody titre dropped significantly from 3.9 (3.7 – 4.1) in 2006 to 3.3 (3.2 – 3.5) in 2007 and again in 2008, to 2.6 (2.3 – 2.8) (p<0.001) (*Figure 5.3*). At Thomsons Lake, the average log transformed neutralising antibody titre also decreased significantly between the time samples were first collected in Apr-June (4.4, 4.3 – 4.6) and when sampling ceased in the Oct-Dec quarter (3.4, 2.9 – 3.9) (p=0.02). Preston Beach was the only location to show a significant increase in the average neutralising antibody titre in kangaroos over time. The average log transformed neutralising antibody titres of positive samples collected between June 2006 and May 2007 was 2.5 (2.0 – 3.1), increasing significantly to 3.7 (3.1 – 4.4) in

December 2007 (p=0.013). Samples were not collected between May and December of 2007 at Preston Beach.

5.3.8. Association Between Accumulated Rainfall and the Average Log Transformed Ross River Virus Neutralising Antibody Titre in Western Grey Kangaroos

All rainfall variables (RainCat30 – RainCat360) were significantly associated with the RRV neutralising antibody titre recorded for kangaroos from all sample collection locations. The significance of the association increased as the length of the preceding period in which accumulated rainfall was calculated over also increased (*Table 5.12*). An analysis of deviance supported this finding, indicating that rainfall in the preceding 360 days explained more of the variance in RRV neutralising antibody titre than any other time period. The average log transformed antibody titres (with 95% confidence intervals) for all collection locations across RainCat360 categories are listed in *Table 5.13*. There was a significant interaction between location and accumulated rainfall (p<0.001), which can be explained by differences in the rainfall category that reported the highest seroprevalence for each location. At Boyup Brook, Capel, Manjimup and Preston Beach, antibody titres were statistically higher following lower periods of accumulated rainfall (p<0.05), whilst at Thomsons Lake, antibody titres increased following higher rainfall (p<0.05). Samples were not collected in all rainfall categories at all locations, making it difficult to assess overall trends at all locations.

Figure 5.3 Temporal changes in seroprevalence and the average log transformed Ross River virus neutralising antibody titre in western grey kangaroos (*Macropus fuliginosus*) at Capel

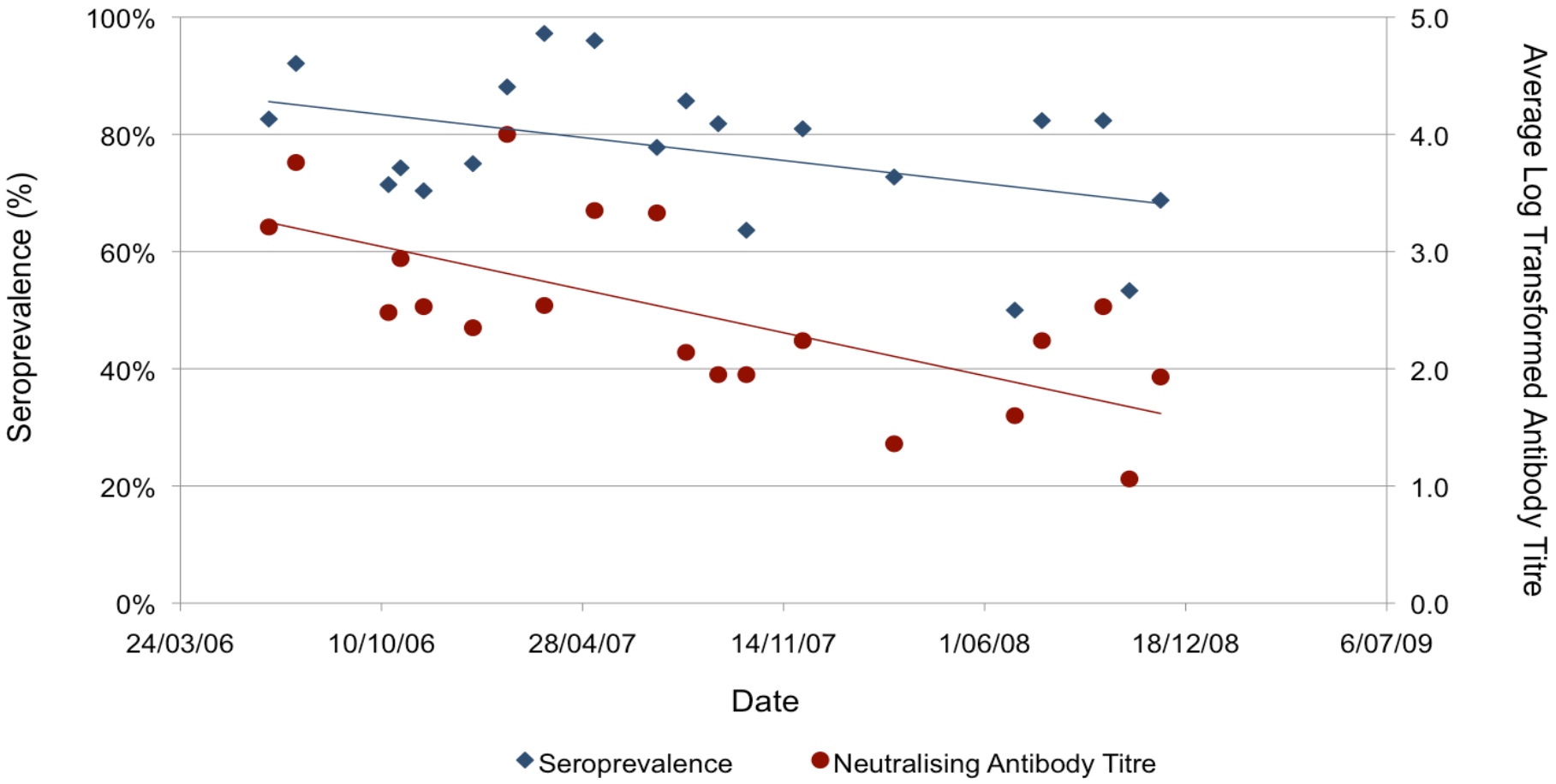


Table 5.12 Significance of association between rainfall variables and the average log transformed Ross River virus neutralising antibody titres across all locations

Accumulated Rainfall Category	*p* Value
RainCat30	0.026
RainCat60	0.003
RainCat90	0.013
RainCat180	< 0.001
RainCat360	< 0.001

Table 5.13 Average log transformed Ross River virus neutralising antibody titre for each RainCat360 category in all sample collection locations

RainCat360	Average Log Transformed Neutralising Antibody Titre (95% CI)			
Location	**< 500mm**	**500-699 mm**	**700-899 mm**	**≥900mm**
Badgingarra	0.2 (0, 0.4)	*NC*	*NC*	*NC*
Balingup	*NC*	*NC*	*NC*	1.2 (0.3, 2.0)
Boyup Brook	1.5 (1.2, 1.9)[a]	0.3 (0, 0.8)[b]	*NC*	*NC*
Bridgetown	*NC*	*NC*	1.1 (0.6, 1.6)	*NC*
Capel	3.9 (3.6, 4.1)[a]	2.9 (2.7, 3.1)[b]	2.6 (2.1, 3.0)[b]	*NC*
Eneabba	0.5 (0.2, 0.9)	*NC*	*NC*	*NC*
Greenbushes	*NC*	*NC*	1.3 (0.6, 2.0)	*NC*
Manjimup	*NC*	*NC*	1.5 (1.2, 1.7)[a]	0.6 (0.2, 1.0)[b]
Myalup	*NC*	*NC*	0.8 (0.5, 1.1)	*NC*
Nannup	*NC*	0.8 (0.2, 1.5)[a]	0.2 (0, 0.8)[a]	*NC*
Northcliffe	*NC*	*NC*	*NC*	0.3 (0, 0.6)
Preston Beach	*NC*	1.6 (1.0, 2.2)[a]	0.2 (0, 0.8)[b]	*NC*
Scott River	*NC*	0.2 (0, 1.2)[a]	2.4 (2.0, 2.9)[b]	1.2 (0.7, 1.8)[a]
Thomsons Lake	4.1 (2.9, 5.3)[a]	5.0 (4.8, 5.3)[a]	5.6 (5.2, 5.9)[b]	6.3 (5.7, 6.8)[c]

NC *Samples not collected in given rainfall category*

a, b, c *Different letters represent statistically significant differences between rainfall variables for a given location at the 5% level of significance.*

5.3.9. Association Between Quarter and the Average Log Transformed Neutralising Antibody Titre in Western Grey Kangaroos

Quarter was significantly associated with the log transformed RRV neutralising antibody titre (p=0.033) across all locations. The magnitude of the association differed depending on the sample collection location (*Table 5.14*). At Manjimup, neutralising antibody titres were significantly lower in the Apr-Jun quarter than all other quarters (p<0.05). At Scott River, a similar trend was noted with neutralising antibody titres significantly lower in Apr-June than in Oct-Dec (p<0.05). At Boyup Brook titres were significantly higher in the Jul-Sep quarter than in either the Apr-June or Oct-Dec quarters (p<0.05). Similar trends were also noted at Bridgetown and Myalup, although these relationships were not statistically significant. At Thomsons Lake, neutralising antibody titres decreased significantly each quarter from the time of first collection in Apr-June until the cessation of sampling in the Oct-Dec quarter of the same year (p<0.05). At Capel, Badgingarra, Eneabba and Preston Beach titres decreased in a similar manner from Jan-Mar through until Oct-Dec, although these trends were not statistically significant over the entire collection period.

Table 5.14 Average log transformed Ross River virus neutralising antibody titre for each quarter in all sample collection locations

	Average Log Transformed Neutralising Antibody Titre (with 95% CI)			
Location	**Jan-Mar**	**Apr-Jun**	**Jul-Sep**	**Oct-Dec**
Badgingarra	0.0 (0, 1.2)[a]	0.4 (0, 0.8)[a]	0.23 (0, 0.6)[a]	0.13 (0, 0.5)[a]
Balingup	*NC*	*NC*	*NC*	1.16 (0.3, 2.0)
Boyup Brook	*NC*	0.71 (0.6, 1.2)[a]	1.93 (0.5, 2.3)[b]	0. 21 (0, 1.0)[a]
Bridgetown	*NC*	0.97 (0.3, 1.6)[a]	1.2 (0.5, 1.9)[a]	*NC*
Capel	3.5 (3.2, 3.8)[a]	3.45 (3.1, 3.8)[a]	3.17 (2.9, 3.4)[a]	2.98 (2.7, 3.3)[a]
Eneabba	*NC*	0.73 (0, 1.6)[a]	0.64 (0.1, 1.2)[a]	0.28 (0, 1.0)[a]
Greenbushes	*NC*	*NC*	1.28 (0.6, 2.0)	*NC*
Manjimup	1.3 (0.8, 1.8)[a]	0.09 (0, 0.7)[b]	1.29 (0.9, 1.7)[a]	1.45 (1.1, 1.8)[a]
Myalup	0.5 (0, 1.3)[a]	0.76 (0.3, 1.2)[a]	1.0 (0.5, 1.5)[a]	0.65 (0, 1.4)[a]
Nannup	0.2 (0, 0.8)[a]	0. 82 (0.2, 1.4)[a]	*NC*	*NC*
Northcliffe	0.3 (0, 0.6)[a]	*NC*	*NC*	0.34 (0, 0.9)[a]
Preston Beach	*NC*	1.81 (1.4, 2.3)[a]	*NC*	1.9 (1.6, 2.3)[a]
Scott River	1.9 (0.7, 3.1)[ab]	0.72 (0, 1.5)[a]	1.66 (0.9, 2.4)[ab]	2.09 (1.7, 2.5)[b]
Thomsons Lake	*NC*	5.64 (5.3, 5.9)[a]	5.15 (4.8, 5.5)[ab]	4.52 (4.0, 5.0)[b]

NC *Samples not collected in given quarter category*

[a, b] *Different letters represent statistically significant differences between quarter categories for a given location at the 5% level of significance.*

5.3.10. Association Between Age and the Average Log Transformed Neutralising Antibody Titre in Western Grey Kangaroos

Sex ($p<0.02$) and age ($p<0.001$) were statistically associated with the log transformed RRV neutralising antibody titre in WGKs. Significantly higher antibody titres were reported in adult and female kangaroos compared to subadult and male kangaroos, respectively ($p<0.05$) (*Table 5.15*).

Table 5.15 Average log transformed Ross River virus neutralising antibody titre for each sex and age group category

Variable	Average Log Transformed Antibody Titre	95% Confidence Interval
Age Group		
Adult	2.8	2.7, 2.9
Subadult	2.1	1.9, 2.3
Sex		
Female	2.9	2.7, 3.0
Male	2.6	2.5, 2.7

5.3.11. Maternal Immunity

A total of 62 paired joey and doe serum samples were collected for this analysis. Of those mothers testing seropositive to RRV neutralising antibodies, 78.5% of their joeys were also seropositive (n=53). Of the nine does testing seronegative, 77.8% of their pouch young were similarly seronegative (n=9). There was a statistically significant, moderate correlation between the two factors (r=0.44, p<0.001). When comparing the average log transformed neutralising antibody titres of the does and their pouch young, there was also a statistically significant, moderate correlation (r=0.43, p<0.001). Interestingly, one doe testing seronegative on the NT contained a pouch young that was seropositive.

5.3.12. Human Attack Rates

The human attack rates for RRV disease, at each of the collection locations, are reported at the Local Government (*Table 5.16*) and suburb/town level (*Table 5.17*). The highest average attack rate over the past five years in both geographic divisions was reported at Preston Beach, followed by Capel. The highest average attack rate across all combined collection locations was reported over the 2005/06 arboviral season. This result was similar at both the Local Government and suburb/town level. No cases of RRV disease were acquired from the Local Government district or suburb/town in which Badgingarra or Eneabba are located during this time.

Table 5.16 Ross River virus disease attack rates reported at the Local Government level for each collection location.

Location	Local Government	Local Government Attack Rates* (%)					Average
		04-05	05-06	06-07	07-08	08-09	
Badgingarra	Dandaragan	0.00	0.00	0.00	0.00	0.00	**0.00**
Balingup	Donnybrook-Balingup	0.02	0.06	0.00	0.04	0.17	**0.06**
Boyup Brook	Boyup Brook	0.06	0.13	0.00	0.00	0.06	**0.05**
Bridgetown	Bridgetown-Greenbushes	0.07	0.12	0.02	0.05	0.21	**0.09**
Capel	Capel	0.00	0.26	0.07	0.11	0.32	**0.15**
Eneabba	Carnamah	0.00	0.00	0.00	0.00	0.00	**0.00**
Greenbushes	Bridgetown-Greenbushes	0.07	0.12	0.02	0.05	0.21	**0.09**
Manjimup	Manjimup	0.03	0.15	0.01	0.05	0.01	**0.05**
Myalup	Harvey	0.02	0.10	0.09	0.06	0.10	**0.07**
Nannup	Nannup	0.08	0.08	0.00	0.08	0.00	**0.05**
Northcliffe	Manjimup	0.03	0.15	0.01	0.05	0.01	**0.05**
Preston Beach	Waroona	0.00	0.50	0.06	0.32	0.18	**0.21**
Scott River	August-Margaret River	0.02	0.06	0.02	0.03	0.09	**0.04**
Thomsons Lake	Cockburn	0.01	0.04	0.02	0.03	0.03	**0.03**
Average		**0.03**	**0.13**	**0.02**	**0.06**	**0.10**	

**Attack rates reported as a percentage of the population residing in the Local Government District in which each collection location is situated.*

Table 5.17 Ross River virus disease attack rates reported at the Suburb level for each collection location.

Location	Suburb /Town	Suburb Attack Rates* (%)					Average
		04-05	05-06	06-07	07-08	08-09	
Badgingarra	Badgingarra	0.00	0.00	0.00	0.00	0.00	**0.00**
Balingup	Balingup	0.00	0.04	0.00	0.00	0.04	**0.02**
Boyup Brook	Boyup Brook	0.00	0.10	0.00	0.00	0.10	**0.04**
Bridgetown	Bridgetown	0.10	0.17	0.00	0.07	0.00	**0.07**
Capel	Capel	0.00	0.47	0.09	0.05	0.14	**0.15**
Eneabba	Eneabba	0.00	0.00	0.00	0.00	0.00	**0.00**
Greenbushes	Greenbushes	0.00	0.00	0.00	0.00	0.00	**0.00**
Manjimup	Manjimup	0.05	0.14	0.02	0.05	0.02	**0.06**
Myalup	Myalup	0.00	0.00	0.00	0.65	0.00	**0.13**
Nannup	Nannup	0.24	0.24	0.00	0.24	0.00	**0.14**
Northcliffe	Northcliffe	0.24	0.24	0.00	0.24	0.00	**0.14**
Preston Beach	Preston Beach	0.00	1.10	0.00	0.00	0.55	**0.33**
Thomsons Lake	Beeliar	0.05	0.07	0.02	0.05	0.02	**0.04**
Average		**0.05**	**0.20**	**0.01**	**0.10**	**0.07**	

**Attack Rates reported as a percentage of the population residing in the Suburb in which each collection location is situated.*

5.3.13. Correlation Between Ross River Virus Disease in Humans and Infection in Kangaroos in Western Australia

There was a significant correlation between the annual attack rate of RRV disease in people, reported at the Local Government level, and both the average log transformed neutralising antibody titre (r=0.542, p=0.004) and seroprevalence (r=0.605, p=0.001) in kangaroos (with a six month lag in kangaroo data) (*Figure 5.4* and *Figure 5.5,* respectively*)*. In this analysis, human case data was aggregated over twelve months, from July 1st through until June 30th of the following year, coinciding with the arboviral season. Kangaroo data was aggregated from January 1st until December 31st and lagged by six months. There was also a significant, but weaker, correlation between the attack rate of RRV disease in people reported at the local government level and the seroprevalence in kangaroos reported over the same period of time (no lag) (r=0.364, p<0.05). There was no correlation between any of the remaining human and kangaroo variables including suburb attack rates and attack rates averaged over a five-year period.

Figure 5.4 Correlation between the annual attack rate of Ross River virus disease in humans, reported at the Local Government level, and the average log transformed neutralising antibody titre in kangaroos (lag period of 6 months in kangaroo data)

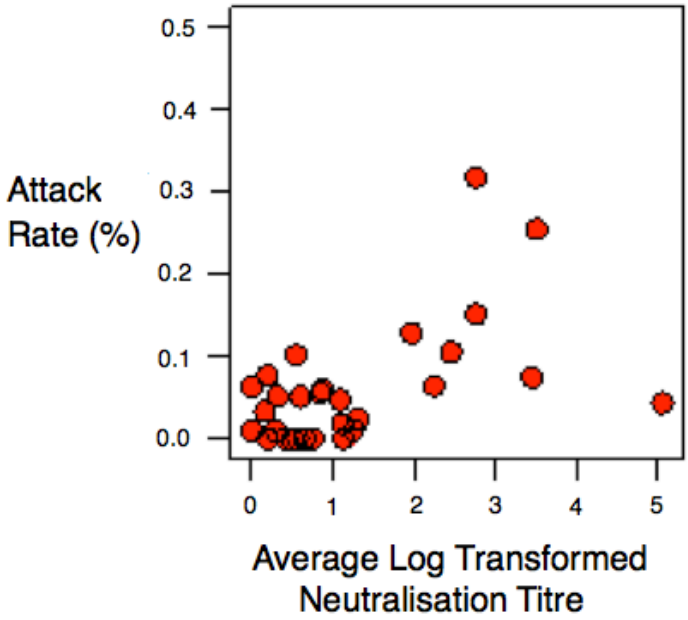
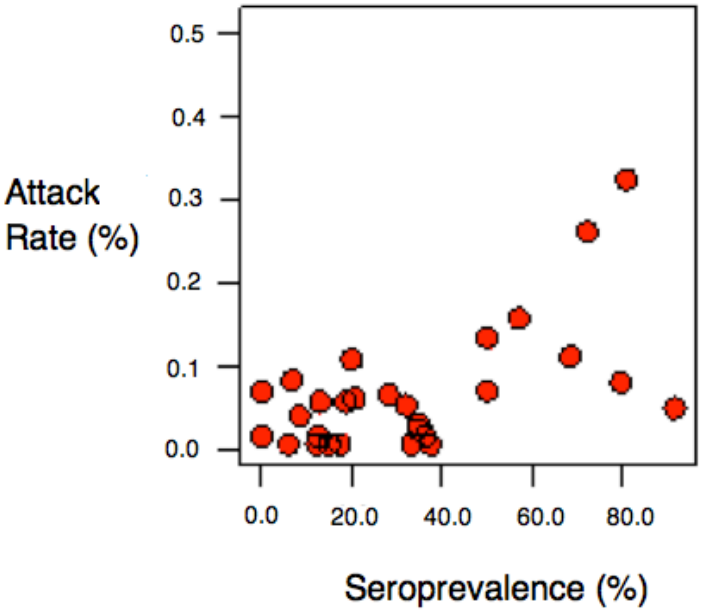


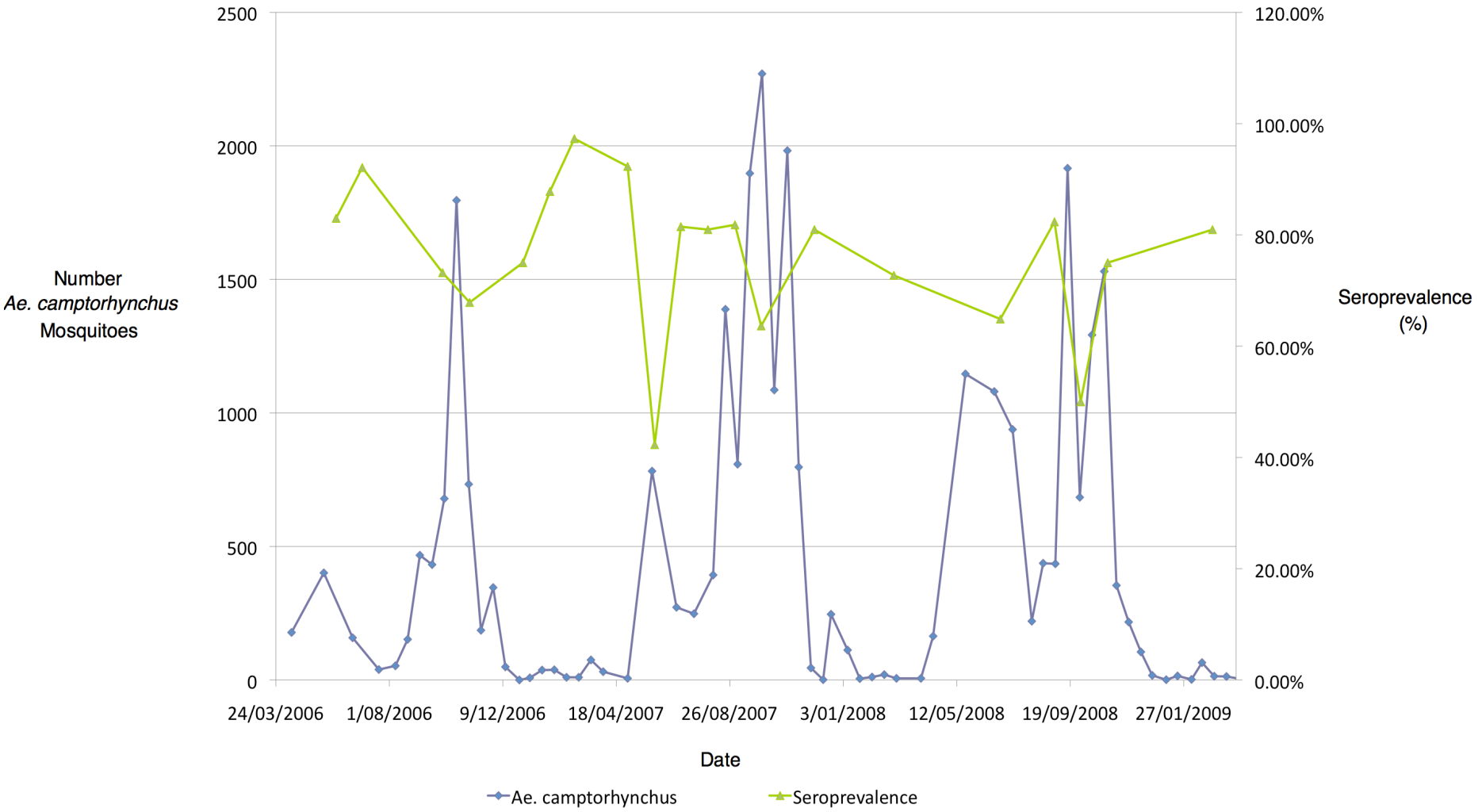
Figure 5.5 Correlation between the annual attack rate of Ross River virus disease in humans, reported at the Local Government level, and the seroprevalence in kangaroos (lag period of 6 months in kangaroo data)



5.3.14. Association Between Local Mosquito Populations and the Seroprevalence of Ross River Virus Neutralising Antibodies in Kangaroos at Capel, Western Australia

There was a significant association between the seroprevalence in kangaroos at Capel and all mosquito population variables ($p<0.001$). These variables included the total number of mosquitoes of all species and the total number of *Ae. camptorhynchus* mosquitoes recovered from the traps in the 14 days and 42 days preceding sample collection from kangaroos. The analysis indicated that the total number of *Ae. camptorhynchus* mosquitoes in the 42 days preceding sample collection explained more of the variance in seroprevalence than all other mosquito population variables (*Figure 5.6*). The regression coefficient for this variable was -0.27 ± 0.07 which indicated that as *Ae. camptorhynchus* mosquito numbers rose, the seroprevalence of RRV neutralising antibodies in kangaroos decreased. There was no association between mean infection rate in mosquitoes and seroprevalence.

Figure 5.6 Total number of *Aedes camptorhynchus* mosquitoes recorded at "CALM Village" and the "Intersection of Stirling and Higgins Road" trap sites compared to the seroprevalence of Ross River virus neutralising antibodies in Capel kangaroos at each collection date



5.3.15. Association Between Age and Sex and the Seroprevalence of Ross River Virus Neutralising Antibodies in Kangaroos at Capel

Sex (p=0.024) and age group (p<0.001) were significantly associated with the seroprevalence of RRV neutralising antibodies in kangaroos at Capel. Seroprevalence was significantly higher in females (78.3%, 67.5 – 76.3) than in males (72.1%, 72.9 – 82.8), and in adults (77.7%, 74.0 – 81.0) compared to subadults (48.0%, 36.6 – 63.4).

5.3.16. Correlation Between Monthly Attack Rates of Ross River Virus Disease in People and both the Seroprevalence and Neutralising Antibody Titre in Kangaroos from Capel

There was no statistically significant relationship between Local Government or suburb/town RRV disease attack rates at Capel and either the average neutralising antibody titre or seroprevalence in kangaroos sampled in the same month, one, two or three months preceding and following sample collection (p>0.05).

5.4. Discussion

5.4.1. Validity of the Data

The results of this study have provided serological evidence that suggests WGKs are commonly infected with RRV in Western Australia. The NT has been the method of choice in a number of previous studies for detecting antibodies to RRV in a wide range of animals (Spradbrow 1972; Gard, Marshall et al. 1977; Cloonan, O'Neill et al. 1982; Vale, Spratt et al. 1991; Lindsay 1995; Agresti and Coull 1998; Boyd, Hall et al. 2001; Boyd and Kay 2002). Whilst not as sensitive as the plaque-reduction neutralisation test, the serum NT is reproducible, relatively inexpensive and makes use of virus-control assays to ensure the virus titre used is accurate (Boyd and Kay 2001). Due to the large number of animals sampled, the NT was the more appropriate choice for antibody detection in this study. The NT was chosen over the haemagglutination-inhibition (HI) because there is considerably less cross reactivity between alphaviruses using the NT (Peters and Dalrymple 1990; Johansen, Mackenzie et al. 2005) and over the plaque reduction neutralisation assay due to its economic advantages. A conservative antibody cut-off titre was selected to ensure that all samples recorded as positive were more likely to be true positives.

5.4.2. The Role of the Kangaroo as Reservoir Host of Ross River Virus in Western Australia

The vertebrate hosts of RRV have not yet been confirmed. However, evidence from previous studies suggests that kangaroos are likely to play a significant role in the amplification and transmission of RRV in Western Australia (Karabatsos 1975; Johansen, Broom et al. 2005). The results of this study support the work undertaken by Lindsay (1995), who showed that 35% of all WGKs across a number of

geographical regions in WA were seropositive for RRV neutralising antibodies. Of the sera collected from 19 different species of non-human vertebrates, the most commonly infected marsupial species was the WGK (Lindsay 1995). In the South Coastal region of WA, the seroprevalence among WGK samples collected during an outbreak of human disease was 87.5% (Lindsay 1995). The results of the study undertaken here show a similarly high seroprevalence amongst kangaroos harvested in Capel and Thomsons Lake. These findings suggest that mosquito vectors capable of infecting vertebrate hosts with the virus commonly feed on macropods in these South Coastal regions of WA where RRV regularly cycles. Bloodmeal analyses undertaken in Western Australia show that putative RRV mosquito vectors, *Ae. camptorhynchus, Ae. vigilax* and *Cx annulirostris*, commonly feed on marsupials (Lindsay 1995).

The role of the WGK as a reservoir of RRV is unclear because of the paucity of data on the magnitude and duration of viraemia following RRV infection. Limited experimental infection studies have demonstrated that eastern grey kangaroos (*M. giganteus*) and agile wallabies (*M. agilis*) develop a viraemia persisting for approximately 3.4 and 6.0 days, respectively, following infection with RRV (Johansen, Power et al. 2009). It is likely that a similar response could be expected in the WGK because these species are closely related. From seven successful attempts to isolate RRV from non-human vertebrate hosts, two have been achieved from agile wallabies (*M. agilis*) (Whitehead, Doherty et al. 1968; Pascoe, St George et al. 1978; Kay, Hall et al. 1986; Harley, Sleigh et al. 2001). By combining the results from all experimental infection, virus isolation and serology studies, many authors have suggested that marsupials are more efficient amplifiers of RRV than other mammals,

which in turn are better than birds (Doherty, Standfast et al. 1971; Kay, Hall et al. 1986; Kay and Aaskov 1989; Harley, Sleigh et al. 2001).

5.4.3. The Association Between Location and the Seroprevalence and Neutralising Antibody Titre of Ross River Virus in Western Grey Kangaroos

The significant difference in both the seroprevalence and the average log transformed neutralising antibody titre in kangaroos between collection locations reflects the geographic variation in RRV activity in Western Australia. Viral transmission is favoured in regions where environmental conditions promote vector abundance and reservoir host numbers are large. Interestingly, the effect of both quarter and rainfall on seroprevalence and the average neutralising antibody titre varied depending on the collection location. These observations are consistent with the findings of Tong and Hu (2002) who reported that climatic variability plays a significant but varying role in the transmission of RRV depending on the location. In particular, there appears to be differences in the response of RRV to variability in climatic factors, such as temperature and humidity, between coastline and inland regions (Marshall and Miles 1984). This may be due to the presence of different vector species in different regions.

Seroprevalence and average neutralising antibody titres for positive samples were higher in kangaroos from Thomsons Lake and Capel than all other collection locations. Given experimental infection studies indicate that RRV antibody titre is highest immediately following seroconversion (Tong and Hu 2002), this finding suggests that there was recent high levels of transmission of RRV. Alternatively, it

may also indicate that transmission was at a stable, low but persistent level within the area the animals were sampled. Reports of above average attack rates of human RRV disease at both locations during the arboviral season that ceased just prior to the beginning of sample collection support the interpretation that a recent epidemic had occurred in both Capel and Thomsons Lake (Kay, Hall et al. 1986). Local Government districts, Capel and Cockburn, also reported additional cases of RRV disease in people in the years following 2006, indicating that viral transmission continued to occur, albeit to a lesser degree, during interepidemic years (Mosquito Borne Disease Control Branch 2009).

Comparatively, the lowest seroprevalence and average neutralising antibody titres for positive samples were reported in kangaroos from Badgingarra and Eneabba. No human cases of RRV disease were reported from either location over the past five years, suggesting that viral transmission in the area is unlikely (Mosquito Borne Disease Control Branch 2009). The results of this study suggest that seroprevalence and average antibody titres in local kangaroos may provide an indication of the background level of risk associated with RRV disease in any given area. This may be used as a risk assessment tool for regions in which little or no mosquito surveillance or human case data are available.

5.4.4. The Association Between Accumulated Rainfall and the Seroprevalence and Neutralising Antibody Titre of Ross River Virus in Western Grey Kangaroos

The significant association between rainfall variables and the seroprevalence and average log transformed titre of RRV neutralising antibodies is difficult to interpret

because seroprevalence cannot typically be used as an indicator of active infection. This finding does warrant further investigation, as rainfall in the preceding months is recognised as the most important risk factor in determining whether a RRV epidemic will occur (Mosquito Borne Disease Control Branch 2009). Rainfall over the preceding one to two months significantly influences the breeding, survival and abundance of mosquito vectors and consequently contributes to the cyclicity of the virus. The activity of other vector-borne viral diseases in Australia, such as Bovine Ephemeral Fever and Bluetongue disease, are similarly influenced by rainfall in this manner (Kelly-Hope, Purdie et al. 2004). Close monitoring of weather patterns is now used to improve the capacity to predict impending RRV activity (Russell 1998; Mackenzie, Lindsay et al. 2000; Woodruff, Guest et al. 2002; Kelly-Hope, Purdie et al. 2004; Woodruff, Guest et al. 2006). This discussion should be considered with caution given seroprevalence does not provide an accurate indication of infection, merely previous exposure to the virus.

The increase in deviance for the rain category variables from RainCat30 to RainCat360 indicated that accumulated rainfall over the 360 days preceding sample collection fitted the statistical model best. Long-term rainfall influences the reproduction of vertebrate hosts by affecting future food supplies (Norbury, Coulson et al. 1988; Arnold, Grassia et al. 1991; Tong and Hu 2001). A decrease in reproduction is commonly associated with drought while an increase in reproduction has been correlated with lagged rainfall (Newsome 1965; 1966; 1977; Bayliss 1985; Arnold, Grassia et al. 1991; Cairns and Grigg 1993; Tong and Hu 2001). Therefore, an increase in long term rainfall will lead to increased numbers of juvenile kangaroos within the environment and a reduction in mob immunity to RRV. In these conditions,

if adequate numbers of mosquito vectors are present simultaneously, there may be an increase in RRV transmission among reservoir hosts and an increased likelihood of a RRV epidemic occurring in human populations.

Interestingly, the significant association between location and RainCat360 suggested that the effect of accumulated rainfall over the 360 days prior to sample collection differed for each location. Whilst similar interactions between climate variables and locality have been noted in RRV studies elsewhere (Norbury, Coulson et al. 1988), the RainCat360 by location interaction was difficult to understand because not all rainfall categories were present at all sites and sampling time frames differed between collection locations. At Thomsons Lake seroprevalence and the average neutralising antibody titre increased following higher rainfall in the 360 days prior to sample collection. This observation supports the hypothesis that long term rainfall leads to increased macropod reproduction, a reduction in mob immunity and above average RRV transmission as an outbreak of RRV in people was reported over the 2005/06 arboviral season. The sampling time frame at Thomsons Lake extended over a period of six months only which needs to be taken into consideration when comparing these findings to those noted at Capel.

In contrast to the findings at Thomsons Lake, the seroprevalence and average neutralising antibody titre in kangaroos in Capel decreased following higher rainfall in the 360 days prior to sample collection. This observation may still support the hypothesis that increased long term rainfall leads to decreased mob immunity and can possibly be explained by differences in the sampling time frame between Capel and Thomsons Lake. Sampling in Capel also began shortly after the 2005/06 arboviral

season but continued over two consecutive interepidemic years. During this time, the seroprevalence and average neutralising antibody titres in kangaroos decreased significantly, reducing mob immunity in the lead up to the next epidemic. The reduction in the mob immunity over this time was the likely result of adequate long-term rainfall leading to increased macropod reproduction.

Given seroprevalence is not an accurate indicator of infection with RRV, this area of research requires further investigation. Sample collection needs to continue in regions where RRV cycles regularly, such as Capel and Thomsons Lake, over a number of epidemic and intervening years. It remains likely that long-term rainfall has a positive influence on food availability, reproduction and therefore the number of potential vertebrate hosts within the environment capable of transmitting RRV.

5.4.5. The Association Between Quarter and the Seroprevalence and Neutralising Antibody Titre of Ross River Virus in Western Grey Kangaroos

The significant association between quarter and both seroprevalence and the average neutralising antibody titre across all collection locations may reflect the seasonal nature of RRV. Viral transmission in the study region is consistently highest between September and April, with the majority of human cases of RRV disease being reported during these months (Tong and Hu 2002; Mosquito Borne Disease Control Branch 2009). Again, this finding requires further investigation and must be considered with caution due to the limitations of using seroprevalence as a predictor of active infection.

The observation that no one quarter was associated with either increasing seroprevalence or antibody titre levels across all sample collection sites was interesting. This can possibly be explained by the month to month changes in climatic and environmental variables that vary between geographic locations. The contrasting patterns of RRV disease transmission between the Southwest and the Kimberley regions of Western Australia provides an extreme example of where this occurs and can be attributed to variation in the timing of risk factors between the two locations (Johansen, Broom et al. 2005). It may also reflect the fact that samples were not collected during an epidemic year. Had samples been collected during a year where RRV activity was significantly above average, quarterly changes in seroprevalence and antibody titre may have been more apparent.

In Capel, the significant increase in seroprevalence between the Oct-Dec and Jan-Mar quarter was likely due to increased transmission of RRV from mosquitoes to kangaroos. Although not statistically significant, the increase in the average log transformed neutralising antibody titre between these two quarters also supports this assumption. Given that higher titres are indicative of a more recent infection (Lindsay, Breeze et al. 2005), the observed increase in seroprevalence in Capel in Jan-Mar was likely due to a period of maximum seroconversion in kangaroos. A similar pattern was also noted at Thomsons Lake where the seroprevalence was statistically lowest in the Oct-Dec quarter and the average neutralising antibody titre decreased in each successive quarter, from Apr-June through until Oct-Dec. These combined observations suggest that viral transmission between mosquitoes and kangaroos occurred at a similar time to people (Kay, Hall et al. 1986). It provides temporal

evidence that kangaroos play an epidemiologically significant role in the transmission of RRV in the southwest of Western Australia.

The remainder of the collection locations demonstrated non-statistical trends between quarter and seroprevalence/neutralising antibody titre, although a number of these supported the observations seen at Thomsons Lake and Capel. Manjimup and Boyup Brook were the only exceptions, reporting statistically lower seroprevalence levels in the Apr-June quarter and higher levels in the Jul-Sept quarter, respectively. The epidemiology of RRV is not well understood in these regions because neither are established RRV foci within Western Australia. Climatic and vector variation may result in differences in the timing and extent of peak transmission periods, accounting for these observations.

The lack of a major epidemic of human RRV disease during the study period suggests that the overall mob immunity in kangaroos at Capel and Thomsons Lake remained sufficiently high to prevent a rapid increase in virus circulation. Alternatively, limiting factors such as climatic conditions, vector abundance or virus virulence, may have played a role in suppressing RRV activity during this time. Sampling needs to continue in the WGK, as well as these other influencing variables, prior to, during and after the next major epidemic to ascertain whether mob immunity plays a significant role in determining the cyclic nature of RRV epidemics. Given RRV cycles every three to four years and the last major period of activity in Capel was in 2005/06, it is likely that the next epidemic will occur in the coming one to two years (Mosquito Borne Disease Control Branch 2009). The average seroprevalence in kangaroos sampled at Capel dropped by approximately ten percent between 2007 and 2008 and

it is likely that a continued reduction in mob immunity will play a critical role in determining whether RRV activity will reach epidemic proportions in future arboviral seasons.

5.4.6. The Association Between Age and the Seroprevalence and Neutralising Antibody Titre of Ross River Virus in Western Grey Kangaroos

The comparison of seroprevalence between adult and subadult kangaroos was complicated because very few subadults were sampled at most collection locations. Conclusions have been drawn from the results at Thomsons Lake and Capel where greater numbers of subadult kangaroos were sampled. In both locations, the seroprevalence of RRV neutralising antibodies in adult kangaroos was statistically higher than in subadults. This finding is consistent with other infectious agents associated with kangaroos, including macropod herpes virus (Mosquito Borne Disease Control Branch 2009), and is due to a combination of repeated exposure to the organism and the possibility that antibodies are retained for a length of time following infection (Kerr, Whalley et al. 1981). Immunocompetent adult kangaroos are unlikely to develop a viraemia of sufficient magnitude and duration to further contribute to viral transmission. Based on this assumption, monitoring changes in seroprevalence in the adult-biased population available through the kangaroo harvesting industry is unlikely to provide an effective means of predicting surges in RRV circulation. This was demonstrated by the lack of correlation between monthly attack rates of RRV disease in people at Capel and changes in the seroprevalence of neutralising antibodies in local kangaroos. However, before this conclusion can be accepted, further surveillance is required to monitor the seroprevalence in kangaroos over a

number of epidemic years. If a significant change in the number of seropositive animals does not precede the increase in reported cases of clinical disease in people during such an episode, it is unlikely that the cost of undertaking such a task would be warranted. It would also be informative to undertake the surveillance in a population consisting of a representative number of subadult kangaroos. Given this is unlikely using the kangaroo harvesting industry, an alternative approach may be to develop a diagnostic assay to detect RRV IgM in adult kangaroos. The NT at present cannot distinguish between a recent or previously acquired infection and although a single IgM assay will not always offer a definitive means of doing so, it is likely to provide more meaningful results from a surveillance perspective. Analysis of the proportion of newly acquired infections will provide a better indicator of current viral activity than simply seroprevalence.

Interestingly, RRV neutralising antibody titres were higher in adult kangaroos compared to subadult kangaroos in this study. This would suggest that adult kangaroos had more recently become infected with the virus than the younger animals or that repeated exposure to the virus over time resulted in higher or more persistent antibody levels. It is possible that this observation was due to the inclusion of negative antibody titres in this analysis. As no significant epidemic occurred during the course of the study, subadult kangaroos probably had less exposure to the virus and therefore were more likely to be seronegative and record lower average antibody titres.

5.4.7. The Association Between Sex and the Seroprevalence and Neutralising Antibody Titre of Ross River Virus in Western Grey Kangaroos

Sex was not significantly associated with seroprevalence, suggesting that both males and females are equally susceptible to being infected with RRV. This is consistent with other infectious agents in macropods, such as macropod herpes virus (Kay, Hall et al. 1986). The observation that female kangaroos reported higher neutralising antibody titres than male kangaroos cannot be explained, particularly given that the seroprevalence was not statistically different between the two sexes.

5.4.8. Correlation Between the Seroprevalence and the Neutralising Antibody Titre of Ross River Virus in Western Grey Kangaroos

The positive correlation between the seroprevalence and average log transformed neutralisation titre provides a potential means of estimating how recently RRV was active within a region. Given that antibody titres peak in macropods within two to four weeks of infection (Kerr, Whalley et al. 1981), high titres are indicative of more recent infection and low titres suggest some time has passed since the last wave of RRV activity. The individual results of this study from each collection location provide evidence to support this conclusion. At Capel and Thomsons Lake, the seroprevalence and average neutralising antibody titre recorded in kangaroos was highest in the initial stages of sample collection in mid-2006. Over time, both seroprevalence and antibody titre decreased. The most recent period of increased viral activity, as evidenced by above average cases of human RRV disease, was reported over the 2005/06 arboviral season for both locations (Kay, Hall et al. 1986). Following this time, no further period of increased viral activity was reported during the course of this study.

The results of this study indicate that following an epidemic, the average seroprevalence and neutralising antibody titres in kangaroos were high, but with time, both continued to decrease. This finding provides evidence that kangaroos play a significant role in determining the cyclicity of RRV in the southwest, where outbreaks tend to occur every three to four years. In some interepidemic years when climatic and environmental conditions favour vector abundance, RRV activity remains at or below average. During other years, epidemics occur following below average rainfall and in the presence of small mosquito populations (Mosquito Borne Disease Control Branch 2009). Whilst both rainfall and vector abundance are considered risk factors in determining whether an outbreak of RRV will occur, it is thought that the abundance of susceptible vertebrate hosts in the environment plays a significant role also (Johansen, Broom et al. 2005). Following an epidemic, a large number of animals will seroconvert as a result of infection. This will limit the number of susceptible hosts remaining within the environment and consequently reduce the likelihood of an RRV epidemic occurring in successive years. Over time, the age structure of the population will change as older animals die and naïve juvenile kangaroos are born. Immunity in individual animals may also wane, reducing the overall mob immunity to RRV (Lindsay, Oliveira et al. 1997). Given the interepidemic period in the southwest is approximately three to fours years, it is plausible that it takes this period of time for the seroprevalence within local kangaroo populations to fall to a sufficiently low level to support another cycle of above average viral activity.

It is currently not known whether waning of individual immunity contributed to the decreasing seroprevalence and average antibody titre over time. The exact length of time in which antibodies persist for in kangaroos is unknown, however, past

experimental infection studies have demonstrated that Murray Valley encephalitis virus HI antibodies in eastern grey kangaroos (*M. giganteus)* circulate for at least 150 days following infection and RRV HI antibodies circulate for at least 168 days in agile wallabies (*M. agilis*) following infection (Kay, Hall et al. 1986). Kay, Hall et al. (1986) noted that with time, the antibody titres in kangaroos did wane, but the study was not continued for long enough to determine whether the animal would eventually become seronegative. Given a reduction in antibody titre is possible, a kangaroo may contribute to transmission of RRV more than once in its own lifetime. Furthermore, the time over which immunity wanes may also contribute to the generation of the three to four year interval between RRV epidemics in the southwest of WA.

5.4.9. The Association Between Mosquito Populations and the Seroprevalence and Neutralising Antibody Titre of Ross River Virus in Western Grey Kangaroos

The statistical association between the seroprevalence of neutralising antibodies in kangaroos at Capel and all variables containing mosquito populations in the preceding periods is consistent with the knowledge that mosquito abundance is considered a major determinant of RRV activity (Kay, Hall et al. 1986; Tong, Hu et al. 2005). Given *Aedes camptorhynchus* is a prominent vector of RRV in the southwest of Western Australia (Woodruff, Guest et al. 2006), it was not surprising that accumulated populations of this species over the 42 days prior to sample collection explained more of the variance in RRV seroprevalence than any other mosquito variable (Ballard and Marshall 1986). The negative relationship between *Ae. camptorhynchus* mosquito populations and seroprevalence however, was an unexpected finding suggesting that the seroprevalence in kangaroos increased as

mosquito numbers were reduced. The seasonal nature of *Ae. camptorhynchus* mosquitoes and the lag period observed in kangaroos between infection and seroconversion may account for this observation. Experimental infection studies showed that kangaroos experience a lag period of approximately two to four weeks between infection and reaching peak antibody titre (Russell 2002). Following a naturally acquired infection, it is possible that this lag period may be longer. Surveillance indicates that *Ae. camptorhynchus* mosquitoes are most abundant in traps between May and November, after which their populations fall rapidly (Kay, Hall et al. 1986). In this study, seroprevalence in kangaroos peaked slightly later than this time period (Jan-Mar). If a large proportion of kangaroos were infected by *Ae. camptorhynchus* in late spring, then it is possible that mosquito populations began to decrease as animals began seroconverting. The negative association between *Ae. camptorhynchus* mosquitoes and the seroprevalence may have also been complicated by the fact that samples were collected during inter-epidemic years where the seroprevalence decreased significantly over the course of the study at Capel. In the past, RRV outbreaks in the southwest of WA have been linked to the persistence of *Ae. camptorhynchus* in the warmer summer months, resulting from rain falling later in the year (Lindsay, Latchford et al. 1989; Arbovirus Surveillance and Research Laboratory 2009). Had samples been collected during an epidemic where this was the case, maximum kangaroo seroconversion is likely to have occurred whilst *Ae. camptorhynchus* mosquitoes were still in abundance.

5.4.10. The Maternal Transfer of Immunity from Doe to Pouch Young

Evidence of maternal transfer of RRV neutralising antibodies between does and their pouch young has been presented in this study, supporting the work of Lindsay (1995)

who detected two seropositive young that were 3 and 6 weeks old, respectively. A number of the seropositive young that were sampled from Capel were naked, pink and had not yet opened their eyes, suggesting they were less than 120 days old (Lindsay, Broom et al. 1992). At such a young age, the pouch young were unlikely to have come into contact with mosquitoes as their heads do not emerge from the pouch until at least day 150 and their first exit is at 298 ± 34 days (Dawson 2002). Maternal transfer of immunity was the most likely explanation for this finding. Despite the acquisition of maternal immunity, antibodies are unlikely to persist for more than 4-6 weeks after the young has left the pouch (Dawson 2002). The joey is therefore able to act as a susceptible vertebrate host of RRV soon after weaning, even in the event that its mother was immunocompetent throughout its pouch life. Fourteen of the 65 pouch young whose mothers tested seropositive for RRV neutralising antibodies, were seronegative. It is possible that a weak immune response in the mother, represented by a low antibody titre, resulted in insufficient maternal immunity acquisition by the pouch young. Sixty-four percent of these young belonged to mothers that had low neutralising antibody titres (40 or 80). In addition, studies have demonstrated that maximum antibody transfer occurs shortly after birth and again during the switch phase, just prior to exiting the pouch (Yadav and Eadie 1973; Deane, Cooper et al. 1990; Adamski and Demmer 1999; Daly, Digby et al. 2007). Immunity may have waned between these two significant periods, corresponding to the time the pouch young were sampled in this study.

5.4.11. Seroprevalence in Kangaroos as an Indicator of the Background Risk of Ross River Virus in a Different Geographic Locations

The observed differences in the average seroprevalence amongst kangaroos in

different geographical locations indicates that exposure to RRV varies from region to region and may provide an indicator of the level of background risk of RRV for any given location. The significant, positive correlation between the annual attack rate of human RRV disease and both the average neutralising antibody titre and seroprevalence in kangaroos (6 month lag period), suggests that patterns of viral activity were similar in kangaroos and humans. The seroprevalence in kangaroos provides a retrospective indicator of the risk of RRV disease for the arboviral season just passed, implying that the relative level of viral activity circulating amongst mosquito vectors and their incidental hosts (people) also occurs in local kangaroo populations. It provides further circumstantial evidence that WGKs are vertebrate hosts of RRV in the southwest of Western Australia, although viraemia studies are required to confirm this.

Interestingly, there was no correlation between the average five-year attack rate and the seroprevalence at either the suburb or local government level. The initial hypothesis of this study was that the overall seroprevalence in local kangaroo populations would provide a means of assessing the background risk of RRV for any given location. Kangaroos from Badgingarra and Eneabba recorded the lowest seroprevalence levels and there were no reported cases of clinical RRV disease in the Local Government district for at least the past five years. In contrast, Capel had the second highest seroprevalence and the second highest average attack rate for human RRV disease out of all study locations (Bell, Stephens et al. 1974). Given these observations, preliminary results appeared to support the hypothesis that seroprevalence in local kangaroo populations could predict the likely background risk of RRV. Despite a lack of statistical correlation, high seroprevalence levels amongst

local kangaroos may still indicate that the risk of RRV is high in a given region, whilst low seroprevalence levels indicate that the risk is low. Estimating the seroprevalence of neutralising antibodies in local kangaroo populations may provide a simple, cost effective means of assessing the background risk of RRV for land-use and urban planning. Current methods are more labour intensive, requiring mosquito trapping and analysis over long periods of time.

5.4.12. Using Seroprevalence and Neutralising Antibody Titres in Kangaroos to Improve the Capacity to Predict Future Ross River Virus Epidemics in People in Capel

In regions where RRV cycles regularly, the seroprevalence of neutralising antibodies in local kangaroo populations will provide an additional dataset that may assist in predicting whether an impending arboviral season is likely to favour above average viral activity. Throughout the duration of this study (June 2006 – November 2008), kangaroos from Capel maintained high average mob immunity and no RRV epidemic was reported. The last major period of viral activity in Capel was during the 2005/06 arboviral season. In 2006 and 2007, the average seroprevalence of neutralising antibodies amongst local WGKs was statistically similar at 72.3% and 78.5%, respectively. In 2008, this percentage dropped significantly to 68.5%. Over the 2008/09 arboviral season, there was a large number of RRV isolates from mosquitoes leading up to and during the peak RRV season, including 16 from the Capel locality (Mosquito Borne Disease Control Branch 2009). Additionally, the average human RRV disease attack rate reported in the Local Government district of Capel rose to its highest level in five years (Arbovirus Surveillance and Research Laboratory 2009). The combination of decreasing seroprevalence towards the end of the sample

collection period and increasing viral activity shortly after, provides evidence to support the notion that vertebrate hosts play a key role in the cyclicity of RRV activity. The results of this study support Johansen et al. (2005) and Lindsay et al. (1997). In 2003/04, a major RRV outbreak occurred despite below average mosquito populations and an absence of usual predisposing climatic and environmental factors (Mosquito Borne Disease Control Branch 2009). It was concluded that the length of time (4 years) since the previous outbreak of RRV in the southwest of WA resulted in larger numbers of susceptible vertebrate hosts within the environment. A low seroprevalence of RRV neutralising antibodies in local WGK populations at Capel (C. Gordon unpublished results) was a likely predisposing factor for high levels of RRV activity (Johansen, Broom et al. 2005). Lindsay (1995) also noted that RRV outbreaks have not been reported from the same meteorological district anywhere in WA in consecutive years, despite favourable environmental conditions occurring during interepidemic years. Whilst yet to be confirmed, inadequate numbers of susceptible vertebrate hosts are considered responsible for suppressing viral activity during these interepidemic years (Lindsay, Breeze et al. 2005). The MBDC and ASRL currently rely on rainfall, tidal activity, temperature, humidity, mosquito population and virus isolation data to make these predictions. This data takes into consideration vector abundance, but ignores the influence of vertebrate host factors on RRV epidemiology. Surveillance of seroprevalence in western grey kangaroos needs to continue over a number of RRV cycles to determine whether a significant drop in seroprevalence is essential for an epidemic to occur, and if so, how low the seroprevalence must fall in local kangaroo populations before above average viral activity will occur.

6. GENERAL DISCUSSION

The knowledge that wildlife species play a significant role in the transmission of emerging infectious diseases was the impetus for this project. A framework for active disease surveillance in kangaroos was developed to assist in the early detection of emerging or exotic diseases and improve the speed of the subsequent response measures put in place to protect both human and animal health. The results of this project demonstrate that the commercial harvesting industry in WA provides a cost effective, efficient means of achieving this goal in kangaroos.

In 2009, 1,950,114 kangaroos were harvested nationally, which provides a substantial sample population for disease monitoring (Lindsay, Oliveira et al. 1997). The sample collection, storage and transport methods developed in this study would be used in a national surveillance program in kangaroos because they are simple, robust and capable of providing samples of a high diagnostic value. Collection of samples at the processor would cost less and permit larger sample sizes, however, the range and quality of specimens that could be collected in this manner is limited. Enlisting the assistance of professional shooters to collect samples shortly after harvesting was shown to be a more a more cost effective than having an investigator travel to remote locations, particularly in large-scale field sampling. This is an important consideration in extending the surveillance framework into New South Wales, Queensland and South Australia, where the majority of human and domestic livestock populations reside and the commercial kangaroo harvesting industry thrives.

Selection bias was identified as the major limitaton of utilising the kangaroo harvesting industry for disease surveillance. However, the harvest figures in WA and Australia are substantial enough that a representative sample of male and female kangaroos could be randomly selected from the harvest population. It is more difficult to overcome the age bias toward adult kangaroos because this is an economic reality in the industry. The impact of selection bias in any specific surveillance program will depend largely on the epidemiological features of the disease/agent in question. In addition, the nature of the diagnostic tools available will also impact the investigators capacity to ameliorate the effect of bias. For example, serological surveillance will be prone to over-estimating the prevalence of an infectious agent because of the trends for older animals to have higher seroprevalence levels. However, it is possible to overcome these problems with careful design of surveillance. Essentially, active disease surveillance in kangaroos obtained through the commercial harvesting industry is considered to be a more effective means of disease detection than traditional, passive surveillance techniques currently adopted in WA. By integrating animal health monitoring with an established wildlife harvesting industry, Western Australia would be better prepared to detect the presence of infectious and zoonotic diseases, reducing the chance of establishment and spread within local kangaroo populations (Department of the Environment Water Heritage and the Arts 2009).

This study has demonstrated that kangaroos are infected with *Salmonella* spp. in their natural habitat. The relatively low prevalence of faecal isolation suggests that wild kangaroos are unlikely to pose any greater risk of zoonotic infection than other livestock species. However, higher levels of carcass contamination reported in previous investigations highlight the need to improve evisceration practices in the

field and hygiene standards at the processor. Particular care should be taken following increased periods of rainfall in the April – June quarter, when prevalence is highest. Whilst kangaroos have not yet been directly linked with food-borne outbreaks of disease, serotypes known to cause severe salmonellosis were isolated from these animals.

Further research is required to characterise the relationship between domestic and wildlife cycles of *Salmonella* in Australia. A longer-term study that includes simultaneous sampling of both kangaroos and local livestock will assist in determining whether transmission occurs between these groups. Given the kangaroo harvesting industry is extensive in SA, QLD and NSW, similar studies should be undertaken in these states to determine whether the prevalence of infection and seasonal patterns are comparable. As carcass contamination provides a potential source of *Salmonella* infection for both pets and humans, it would be sensible for those in contact with kangaroos to practice good hygiene at all times.

Few studies have investigated the role of macropods in the maintenance and transmission of *C. burnetii*. The results of this research suggest that kangaroos are likely to be reservoirs of the organism in Western Australia, posing a zoonotic threat to industry workers and animal handlers. The high level seroprevalence across a number of geographic regions, possibly following periods of increased rainfall, suggests that this finding is wide spread throughout the state. Further research is required to determine the prevalence of *C. burnetii* in kangaroos in other states of Australia. This information is required to ensure that vaccination programs to prevent Q fever in kangaroo shooters and harvesting industry workers are enhanced. It is also

important to determine whether transmission of *C. burnetii* occurs commonly between kangaroos and local livestock species to beter understand the role each species plays in the epidemiology of Q fever in humans.

The results of this study provide evidence to suggest that WGKs play a significant role in the transmission of RRV in the southwest of Western Australia. Routine monitoring of the neutralising antibody seroprevalence in kangaroos in the manner undertaken in this study is unlikely to improve the capacity to predict impending outbreaks of disease in people. This is largely due to the age-based selection bias present within the industry and the increased likelihood that an adult animal is already positive from a previous infection. Given that sampling of kangaroos via the harvesting industry provides one of the only ethical and practical methods of surveillance in these free-living animals, the development of a diagnostic assay to selectively identify IgM is considered a research priority. Detection of a sudden increase in the number of recent infections in adult kangaroos is likely to assist the WA Department of Health in predicting when viral transmission is high and an epidemic in people is likely. Using the current sampling methods and neutralisation test, seroprevalence estimates may still be useful in providing a general indication of the risk of RRV within a given region.

7. APPENDICES

APPENDIX A Request for kangaroo shooter assistance in blood sample collection

Division of Health Sciences

School of Veterinary & Biomedical Sciences

MURDOCH UNIVERSITY
PERTH, WESTERN AUSTRALIA

Postgraduate Student
South Street, Murdoch
a.bestall@murdoch.edu.au
Office (08) 9360 2658

November 2nd, 2006

DISEASE SURVEILLANCE IN KANGAROOS USING THE HARVESTING INDUSTRY

Dear Kangaroo Shooter,

My name is Abbey and I am writing to you today to request your help. As a Veterinarian, I am a strong supporter of your industry and have just begun a research project concentrating on diseases in kangaroos. After speaking to both roo shooters and processing plant workers, I have identified the need to find out more on the following areas:

1. Diseases passed from kangaroos to people (eg. Ross River virus & Q-Fever).
2. Diseases within kangaroo populations (eg. kangaroo blindness).
3. Preventing new diseases being introduced from overseas and interstate to kangaroos in WA.

I am looking for shooters who will be willing to collect blood samples for this project. I will supply collection kits, organise transport back to Perth and cover all costs. Finally, I have included a short survey that I hope you will fill in, regardless of whether you are able to help or not. As my project is based around getting kangaroo shooters to collect blood for disease surveillance, I would like to understand why they

do or do not wish to participate. Please leave your name and contact details blank if preferred. A stamped, addressed envelope is included for survey return.

My supervisor is Associate Professor Stan Fenwick of the Division of Veterinary and Biomedical Sciences. Both he and I are happy to discuss with you any concerns you may have on how this study has been conducted. If you wish to talk to an independent person about your concerns you can contact Murdoch University's Human Research Ethics Committee on 9360 6677. Please be aware that this is a voluntary request, and shooters are not required to participate.

Thank you for taking the time to read this letter. Any help is greatly appreciated and I will keep you up to date with the results of the research. Please feel free to contact me directly or fill in the appropriate section on the survey if you wish for me to contact you.

Sincerely,
Abbey Bestall.

_____

APPENDIX B Questionnaire and Request for Expressions of Interest in Assistance in Kangaroo Disease Surveillance

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1. *Personal Details*:

(Leave NAME blank to remain anonymous)

Name: _____

Where do you live (nearest Town or postcode):

_____

Where do you regularly shoot (nearest Town):

_____

Are you a Full time/Part time shooter:

_____

Main Occupation (other than roo shooting):

_____

Length of time in the roo shooting industry:

_____

Average No. kangaroos shot per night:

- Summer (Dec – Feb): _____
- Autumn (Mar – May) : _____
- Winter (June – Aug): _____
- Spring (Sept – Nov): _____

Average No. nights per month spent shooting:

_____

Name of Processor where you send roos:

_____

2. *What is the main reason you shoot roos?*

(Please tick ONE of the following)

- o Recreation
- o Income
- o Consider kangaroos a pest
- o Other (please specify)

  _____

3. *Can you help to collect blood samples?*

(Please tick ONE of the following)

- o Yes (please go to 4.)
- o Would like to know more (Please go to 4.)
- o No (please got to 5.)

4. *Contact*

- o Shooter to contact Abbey (see contacts below)
- o Abbey to contact Shooter (place details below)

Best way to contact investigator:

Time of day: _____
Email: _____
Work Phone: _____
Mobile No: _____
Home Phone: _____

5. *Reason for not collecting blood:*

(Please tick ONE of the following)

- o Project not of interest
- o Unable to shoot regularly/enough roos to meet project requirements
- o Do not have enough time
- o Other (please specify)

  _____

6. *How long do you plan on shooting kangaroos in your current area?*

- o Less than 1 year
- o 1-2 years
- o 2-3 years
- o Longer than 3 years
- o Unsure

7. *Which disease/s are you aware of in kangaroos?*

(Please tick ONE OR MORE of the following)

- o Q-Fever
- o Salmonella
- o Ross River virus
- o Knee Worms
- o Intestinal Worms
- o Lumpy Jaw
- o Toxoplasma
- o Kangaroo blindness
- o Ticks

8. *Which of the following do you consider most important?*

(Please tick ONE of the following)

- o Monitoring diseases affecting kangaroos
- o Monitoring diseases that can be passed from kangaroos to people
- o Monitoring diseases that can be passed from kangaroos to livestock
- o Preventing new diseases from being introduced into Western Australia
- o Other (please specify) _____

Signature: _____
Date: _____

Please return via enclosed envelope to:

Abbey Bestall (Postgraduate Candidate)
School of Vet & Biomedical Science
South Street, Murdoch
Western Australia 6150

Thank you for completing the survey.
You have greatly contributed to the success of this project.

Please feel free to contact Abbey Bestall if you have any further questions
Email: a.bestall@murdoch.edu.au *Office*: (08) 9360 2658 *Mobile*: 0402 482 743

APPENDIX C Introductory and thankyou letter to participating kanagaroo shooters

Disease surveillance in kangaroo populations in Western Australia

Thank you for agreeing to participate in this project. Commercial shooting provides an opportunity to collect samples for research that would otherwise be impossible. Through your assistance we will develop methods of sample collection in kangaroo populations specific to Western Australia, and in turn, provide an efficient means of disease surveillance. This is a vital area of research as many of Australia's most significant emerging diseases originate from within wildlife populations.

In order to complete the research, I am hoping to obtain blood samples from kangaroos in representative target areas across Western Australia. Blood can be easily collected using the kits provided and all costs will be covered by the research program. Any whole blood that is not chilled within 12 hrs, or alternatively, a chilled sample that must be stored for more than 48 hrs is likely to be non-diagnostic. Please be sure to discuss this with us if you feel it will be a problem. Additionally, to ensure that we are expecting your samples and prepare for testing, please contact Abbey prior to sample collection.

Please be sure to give any feedback on ways to make sample collection easier for you, as this all forms part of my research. Thank you once again for assisting in the success of this project. For further information or to obtain equipment for sampling, please feel free to contact:

Abbey Bestall

Dept of Veterinary & Biomedical Science
Murdoch University
South Street, Murdoch, WA 6150
Ph: 0402 482 743 or (08) 9360 2658
Email: a.bestall@murdoch.edu.au

APPENDIX D Kangaroo blood sampling instructions

SAMPLING PROCEDURE

1 Kit Contents

- ✓ Instructions
- ✓ Ice brick
- ✓ Foam esky
- ✓ Blood tubes
- ✓ Foam tube storage rack
- ✓ Permanent marker
- ✓ Zip-lock bags
- ✓ ID tags

2 Sampling Method

1. Blood Tube

- Label tube with permanent marker ***before*** filling with blood.
- After hanging kangaroo upside down on truck, remove head or cut neck.
- Remove cap and fill tube with blood.
- If possible, collect blood from a stream that is not in contact with the animal's coat.
- Ideally, tubes should be filled between ½ - ¾.
- Store blood tubes upright in rack.
- Place ID TAG onto roo to ensure matching of faecal samples.

- ***Tubes must be chilled in fridge or placed in esky with an ice brick at the end of the shooting run.***
- Please do not ***FREEZE*** samples.

3 Label Instructions

Using a permanent marker, label tubes individually as below.

- **1. Number:**
  Number tubes consecutively. Ensure an ID tag with the same number is placed on the roo to allow matching of faeces at gutting.
- **2. Gender:**
  Simply write an **M** or an **F** on tube
  M – Male
  F – Female
- **3. Age**
  Please estimate age on tube.
  **It becomes difficult to estimate older animals. If older than 5, just write 5+*

  If you are unsure, use **P, SA** or **A**

  P – Pouch Young
  SA – Subadult (<3yrs; not mature).
  A – Adult

4 Faecal Samples

- When removing intestines, leave **2-5** pellets of faeces in the rectum.
- Squeeze 2 pellets of faeces into a zip-lock bag and seal.
- Write the same ID number onto the sample bag that is attached to the roo, to allow blood matching.
- Store faecal samples in esky.

***NOTE**: It is **NOT** necessary to write the age and gender on the faecal bags if they are on the matching blood tubes.*

<u>Thank you for participating in this important research</u>.
If you are unsure of any points in the instructions above, please contact Abbey Bestall on 0402 482 743 prior to collecting samples.

APPENDIX E Recipes for media solutions used in cell culture

Media below was produced using filtered, double distilled water (Millipore Q ultra-filtration) and stored at 4ºC unless otherwise stated, according to the recipes detailed below.

- **M199 Stock Solution**

M199 Powder	11g/L
$NaHCO_3$	0.35g/L
HEPES	4.77g/L
Benzyl penicillin	100mg/L
Gentamycin	10mg/L

The ingredients were dissolved in H_2O and the pH adjusted to 7.4 using NaOH. The solution was sterilised using vacuum filtration.

- **L-Glutamine**

L-Glutamine	14.6g/L
ddH_2O	250ml

The solution was sterilised by vacuum filtration, aliquoted into 10ml volumes and stored at -20ºC.

- **Growth Medium (5% FBS)**

M199 stock solution	500ml
L-Glutamine stock	10ml
FBS	25ml

- **Maintenance Medium (2% FBS)**

M199 stock solution	500ml
L-Glutamine stock	10ml
FBS	10ml

- **Blank Medium (0% FBS)**

M199 stock solution	500ml
L-Glutamine stock	10ml

APPENDIX F Total number of *Aedes Camptorhynchus* mosquitoes, Ross River virus isolates and the minimum infection rate per (MIR) 1000 mosquitoes from the "CALM Village" trap site (Arbovirus Surveillance and Research Laboratory 2009)

Date	No. Mosquitoes	No. RRV isolates	MIR/1000 Mosquitoes
03/01/2006	33	0	0
17/01/2006	0	0	0
02/02/2006	0	0	0
13/02/2006	0	0	0
28/02/2006	0	0	0
14/03/2006	2	0	0
29/03/2006	4	0	0
11/04/2006	22	0	0
18/05/2006	110	0	0
20/06/2006	85	0	0
20/07/2006	19	0	0
08/08/2006	21	0	0
22/08/2006	62	0	0
05/09/2006	183	0	0
19/09/2006	146	0	0
03/10/2006	524	0	0
17/10/2006	1097	0	0
31/10/2006	565	0	0
14/11/2006	61	0	0
28/11/2006	125	0	0
12/12/2006	6	0	0
28/12/2006	0	0	0
09/01/2007	2	0	0
23/01/2007	2	0	0
06/02/2007	0	0	0
20/02/2007	1	0	0
06/03/2007	2	0	0
20/03/2007	4	0	0
03/04/2007	2	0	0
01/05/2007	3	0	0
29/05/2007	255	0	0
26/06/2007	242	0	0
16/07/2007	79	0	0

APPENDIX F cont. Total number of *Aedes Camptorhynchus* mosquitoes, Ross River virus isolates and the minimum infection rate (MIR) per 1000 mosquitoes from the "CALM Village" trap site (Arbovirus Surveillance and Research Laboratory 2009)

Date	No. Mosquitoes	No. RRV isolates	MIR/1000 Mosquitoes
07/08/2007	323	0	0
21/08/2007	584	0	0
04/09/2007	480	0	0
18/09/2007	909	0	0
02/10/2007	816	0	0
16/10/2007	762	0	0
31/10/2007	724	0	0
13/11/2007	423	0	0
27/11/2007	44	0	0
11/12/2007	1	0	0
20/12/2007	75	0	0
08/01/2008	15	0	0
22/01/2008	3	0	0
05/02/2008	5	0	0
19/02/2008	0	0	0
04/03/2008	0	0	0
18/03/2008	8	0	0
01/04/2008	2	0	0
15/04/2008	16	0	0
22/05/2008	612	0	0
24/06/2008	638	0	0
15/07/2008	585	0	0
06/08/2008	131	0	0
19/08/2008	127	0	0
02/09/2008	327	0	0
16/09/2008	998	0	0
30/09/2008	485	5	17.9
14/10/2008	1072	0	0
28/10/2008	344	0	0
11/11/2008	226	5	31.1
25/11/2008	150	0	0
09/12/2008	60	0	0
22/12/2008	17	0	0

APPENDIX G Total number of *Aedes Camptorhynchus* mosquitoes, Ross River virus isolates and the minimum infection rate (MIR) per 1000 mosquitoes from the "Intersection of Stirling-Higgins Road" trap site (Arbovirus Surveillance and Research Laboratory 2009)

Date	No. Mosquitoes	No. RRV isolates	MIR/1000 Mosquitoes
03/01/2006	50	0	0
17/01/2006	32	0	0
02/02/2006	16	0	0
13/02/2006	56	0	0
28/02/2006	4	0	0
14/03/2006	0	0	0
29/03/2006	0	0	0
11/04/2006	156	0	0
18/05/2006	291	0	0
20/06/2006	73	0	0
20/07/2006	20	0	0
08/08/2006	32	0	0
22/08/2006	90	0	0
05/09/2006	284	0	0
19/09/2006	286	0	0
03/10/2006	155	0	0
17/10/2006	699	0	0
31/10/2006	168	0	0
14/11/2006	125	0	0
28/11/2006	221	0	0
12/12/2006	43	0	0
28/12/2006	0	0	0
09/01/2007	6	0	0
23/01/2007	35	0	0
06/02/2007	38	0	0
20/02/2007	9	0	0
06/03/2007	8	0	0
20/03/2007	71	0	0
03/04/2007	29	0	0
01/05/2007	3	0	0
29/05/2007	527	0	0
26/06/2007	30	0	0
16/07/2007	169	0	0

APPENDIX G cont. Total number of *Aedes Camptorhynchus* mosquitoes, Ross River virus isolates and the minimum infection rate (MIR) per 1000 mosquitoes from the "Intersection of Stirling and Higgins Road" trap site (Arbovirus Surveillance and Research Laboratory 2009)

Date	No. Mosquitoes	No. RRV isolates	MIR/1000 Mosquitoes
07/08/2007	70	0	0
21/08/2007	804	0	0
04/09/2007	328	0	0
18/09/2007	988	0	0
02/10/2007	1454	0	0
16/10/2007	324	0	0
31/10/2007	1258	0	0
13/11/2007	374	0	0
27/11/2007	1	0	0
11/12/2007	0	0	0
20/12/2007	171	0	0
08/01/2008	97	0	0
22/01/2008	2	0	0
05/02/2008	6	0	0
19/02/2008	20	0	0
04/03/2008	6	0	0
01/04/2008	4	0	0
15/04/2008	148	0	0
22/05/2008	534	0	0
24/06/2008	442	0	0
15/07/2008	353	0	0
06/08/2008	89	0	0
19/08/2008	310	0	0
02/09/2008	108	0	0
16/09/2008	918	0	0
30/09/2008	199	0	0
14/10/2008	220	0	0
28/10/2008	1186	0	0
11/11/2008	128	0	0
25/11/2008	67	0	0
09/12/2008	45	0	0
22/12/2008	0	0	0

APPENDIX H Total number of mosquitoes, Ross River virus isolates and the minimum infection rate (MIR) per 1000 mosquitoes from the "CALM Village" trap site (Arbovirus Surveillance and Research Laboratory 2009)

Date	No. Mosquitoes	No. RRV isolates	MIR/1000 Mosquitoes
3/01/2006	65	0	0
17/01/2006	17	0	0
02/02/2006	8	0	0
13/02/2006	18	0	0
28/02/2006	10	0	0
14/03/2006	2	0	0
29/03/2006	11	0	0
11/04/2006	23	0	0
18/05/2006	112	0	0
20/06/2006	87	0	0
20/07/2006	20	0	0
08/08/2006	24	0	0
22/08/2006	76	0	0
05/09/2006	191	0	0
19/09/2006	152	0	0
03/10/2006	553	0	0
17/10/2006	1097	0	0
31/10/2006	580	0	0
14/11/2006	72	0	0
28/11/2006	167	0	0
12/12/2006	19	0	0
28/12/2006	2	0	0
09/01/2007	4	0	0
23/01/2007	4	0	0
06/02/2007	4	0	0
20/02/2007	4	0	0
06/03/2007	6	0	0
20/03/2007	7	0	0
03/04/2007	6	0	0
01/05/2007	3	0	0
29/05/2007	265	0	0
26/06/2007	248	0	0
16/07/2007	80	0	0
07/08/2007	332	0	0

APPENDIX H cont. Total number of mosquitoes, Ross River virus isolates and the minimum infection rate (MIR) per 1000 mosquitoes from the "CALM Village" trap site (Arbovirus Surveillance and Research Laboratory 2009)

Date	No. Mosquitoes	No. RRV isolates	MIR/1000 Mosquitoes
21/08/2007	605	0	0
04/09/2007	482	0	0
18/09/2007	929	0	0
02/10/2007	854	0	0
16/10/2007	832	0	0
31/10/2007	824	0	0
13/11/2007	654	0	0
27/11/2007	159	0	0
11/12/2007	71	0	0
20/12/2007	112	0	0
08/01/2008	20	0	0
22/01/2008	5	0	0
05/02/2008	2	0	0
19/02/2008	5	0	0
04/03/2008	0	0	0
18/03/2008	8	0	0
01/04/2008	2	0	0
15/04/2008	26	0	0
22/05/2008	724	0	0
24/06/2008	770	0	0
15/07/2008	635	0	0
06/08/2008	146	0	0
19/08/2008	134	0	0
02/09/2008	334	0	0
16/09/2008	1074	0	0
30/09/2008	529	5	15.9
14/10/2008	1212	0	0
28/10/2008	388	0	0
11/11/2008	314	5	19.6
25/11/2008	196	0	0
09/12/2008	84	0	0
22/12/2008	25	0	0

APPENDIX I Total number of mosquitoes, Ross River virus Isolates and the minimum infection rate (MIR) per 1000 mosquitoes from the "Intersection of Stirling and Higgins Road" trap site (Arbovirus Surveillance and Research Laboratory 2009)

Date	No. Mosquitoes	No. RRV isolates	MIR/1000 Mosquitoes
03/01/2006	59	0	0
17/01/2006	49	0	0
02/02/2006	31	0	0
13/02/2006	87	0	0
28/02/2006	10	0	0
14/03/2006	1	0	0
29/03/2006	1	0	0
11/04/2006	171	0	0
18/05/2006	295	0	0
20/06/2006	78	0	0
20/07/2006	22	0	0
08/08/2006	36	0	0
22/08/2006	100	0	0
05/09/2006	289	0	0
19/09/2006	295	0	0
03/10/2006	179	0	0
17/10/2006	711	0	0
31/10/2006	174	0	0
14/11/2006	161	0	0
28/11/2006	240	0	0
12/12/2006	45	0	0
28/12/2006	0	0	0
09/01/2007	7	0	0
23/01/2007	39	0	0
06/02/2007	46	0	0
20/02/2007	74	0	0
06/03/2007	53	0	0
20/03/2007	86	0	0
03/04/2007	38	0	0
01/05/2007	8	0	0
29/05/2007	454	0	0
26/06/2007	30	0	0
16/07/2007	172	0	0
07/08/2007	78	0	0

APPENDIX I cont. Total number of mosquitoes, Ross River virus isolates and the minimum infection rate (MIR) per 1000 mosquitoes from the "Intersection of Stirling and Higgins Road" trap site (Arbovirus Surveillance and Research Laboratory 2009)

Date	No. Mosquitoes	No. RRV isolates	MIR/1000 Mosquitoes
21/08/2007	815	0	0
04/09/2007	333	0	0
18/09/2007	1022	0	0
02/10/2007	1475	0	0
16/10/2007	344	0	0
31/10/2007	1399	0	0
13/11/2007	486	0	0
27/11/2007	5	0	0
11/12/2007	16	0	0
20/12/2007	299	0	0
08/01/2008	106	0	0
22/01/2008	9	0	0
05/02/2008	17	0	0
19/02/2008	21	0	0
04/03/2008	17	0	0
01/04/2008	11	0	0
15/04/2008	193	0	0
22/05/2008	601	0	0
24/06/2008	461	0	0
15/07/2008	368	0	0
06/08/2008	97	0	0
19/08/2008	314	1	3.3
02/09/2008	110	0	0
16/09/2008	928	0	0
30/09/2008	212	0	0
14/10/2008	242	0	0
28/10/2008	1285	2	5.7
11/11/2008	155	0	0
25/11/2008	85	0	0
09/12/2008	53	0	0
22/12/2008	4	0	0

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